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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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REVIVED ACTIVITY OF THE VIRUS OF POLIOMYELITIS.

By SIMON FLEXNER, M.D., and HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 19, 1923.)

In course of the experimental studies of poliomyelitis carried out during the past 10 years, the varieties and limits of activity of the virus itself have been given little more than passing attention. Although it was early found that not all specimens of the central nervous tissues taken post mortem from acute cases of poliomyelitis equally induced the experimental disease in monkeys, the distinctions in activity have never been accurately defined. The statement that about half the specimens are inoculable upon monkeys is merely a rough approximation, since by increasing the number of monkeys injected, the percentage of successful transmissions is made to rise. There is, however, agreement on the fundamental points, namely that certain specimens are more readily inoculable than others and certain individuals among the susceptible simian species are more easily infected than others.

There is agreement on a third fundamental point. When the human virus of poliomyelitis is first implanted on the monkey, it is less active or virulent than it becomes after several successive implantations or transfers. At the outset the proportion of inoculated monkeys escaping infection, and, having become infected, surviving the disease, is higher than later when, as we say, the virus has become adapted to the new species.

It is obvious that under strictly natural conditions, the monkey is far less susceptible to infection with the virus of poliomyelitis than is man. No instance is known of accidental or as we say "spontaneous" communication of poliomyelitis to the monkey. The recently inoculated and infected animals can be kept in closest contact with healthy and uninoculated companions, without the latter ever acquiring the infection. The protective or defensive mechanisms which the monkey possesses must be circumvented or overcome, as by direct artificial inoculation, in order that infection may ensue.

And yet the experimental disease in the monkey, once it is induced, is more fatal than the original disease in man. An adapted virus commonly infects all the inoculated animals, and of the infected, few if any tend to survive. Histological examination discloses, as a rule, extensive infiltrative lesions of the floor of the fourth ventricle, to which the death of the animal is readily and perhaps properly ascribable.

This remarkably high mortality rate can to a small extent be mitigated by nursing. It is of course difficult to care for severely paralyzed monkeys as severely affected human beings are cared for. However, when great care and patience are exercised with feeding, bedding, etc., occasionally a seriously paralyzed animal may be brought safely through the acute stages of the disease and started on the course of convalescence and recovery. This recovery is rarely complete; although remarkable restorations of function occur in the affected muscles, residual paralyses practically always remain. In this respect there is further analogy between the epidemic disease in man and the experimental disease in monkeys.

Certain human strains of the virus, as indicated, either do not induce the experimental disease at all or quickly lose the power of infection and thus fail to become adapted to the monkey. This loss in power when it occurs tends to be complete. That is, a weak strain tends not to provoke a mild infection; it produces no infection at all. This point is determinable by reinoculation or by neutralization tests. Animals which have recovered from paralyses are immune and their blood serum is neutralizing to the most potent virus, while those receiving the weak strains without exhibiting symptoms react to the powerful strain as do previously uninoculated monkeys.

No readily applicable method is known for cultivating the virus of poliomyelitis outside the body so that its properties may be studied in detail. It is, however, possible to cultivate the virus artificially *in vivo* in the monkey and thus to determine certain of its effects. As has been stated, such successive transfers establish the virus at a high level of activity. Hence the question arises whether this high level will be maintained over a long period and through an indefinite series of animal passages.

The view has been held that certain epidemic diseases in man attain malignancy through successive direct, rapid passages of the inciting microbes from individual to individual. In this way it has been sought to account for the sinister character of institutional outbreaks of streptococcus and pneumococcus pneumonia and the general outbreaks of epidemic influenza. Since no intermediate means are known of the transport of the virus of poliomyelitis from person to person, it is this successive direct mode of communication which has been charged with the initiation of destructive epidemics of the disease.

Epidemics are characterized by movements in two opposite directions: they not only tend to rise from a mean and reach a peak of prevalence or incidence, but they tend also to fall from this peak to or below the mean when the epidemic wave of incidence has spent itself. This is the case no matter whether subsequent rises and falls do or do not take place. If, therefore, the successive passage from individual to individual serves to elevate the activity of the inciting microbe, a point should ultimately be reached when this same process serves to depress the activity in order to account for the phenomena observed.

It is not our purpose to pursue this theme in detail. Our object in this paper is merely to describe a specimen of the virus of poliomyelitis which has been observed to pass through three distinct phases of activity or virulence. The correlation of the facts with the views currently held on the epidemiology of poliomyelitis will not be undertaken at this time.

The specimen of virus to be described was obtained in 1909. It is denominated M. A. It consisted originally of central nervous tissues taken from a fatal case of epidemic poliomyelitis in a child.[1] The implantation on *Macacus rhesus* monkeys offered no special difficulty; the activity at first was not high, but rose quickly on successive transfer. At the outset occasionally a paralyzed animal recovered; later, recovery almost never occurred. The effective infecting dose of the virus fell with the enhancement of virulence. At the period of maximal activity it was 0.01 to 0.001 cc. of a 5 per cent emulsion centrifuged and passed through a Berkefeld filter, given by intracerebral injection.

[1] Flexner, S., and Lewis, P. A., *J. Exp. Med.*, 1910, xii, 227.

Maximal virulence of the strain was maintained for a period of about 3 years, that is from the autumn of 1909 until the winter of 1912, during which it was passed through many monkeys. In the winter of 1912 a change became apparent, consisting in failure at times of a given dose to induce infection. Along with this change another was noted; now and then an animal developing paralysis would recover. The deterioration of the virus continued until it was reestablished at about the level of strength which was exhibited at the original passages made in 1909.[2]

Efforts to augment the virus by more rapid animal passages were unsuccessful; the lower level of activity appeared to be fixed. In this important respect the deteriorated monkey strain of virus differed from the original human strain from which it was derived. Moreover, the debasement did not affect one sample only of the virus, but affected all samples, the offspring of the original material.[2]

The sample or strain of M. A. virus we are considering has now undergone another alteration. It has entered on a new phase of enhanced activity; that is, its virulence has been restored and its high power of inducing infection revived.

In order to follow these new events it is necessary to recall our custom to place in 50 per cent neutral glycerol and to preserve at 4°C. samples of the spinal cord and brain of all animals succumbing to poliomyelitis. In this way a complete series of glycerolated specimens is collected. It had been our belief, based on previous observation, that glycerolation does not modify the virus, but rather preserves it over several years essentially unchanged in activity. In accordance with the custom, samples of the earlier potent and of the later deteriorated M. A. virus were thus set aside. As was stated in the earlier publication describing the deterioration, the question was raised "whether after a resting period a second enhancement can be accomplished by a new series of passages."[2] This question can now be answered in the affirmative.

The second rise in virulence of the M. A. strain was noted in 1918. Between 1912, at which time the reversion took place, and 1918, when high activity was again noticed, the strain was passed through an

[2] Flexner, S., Clark, P. F., and Amoss, H. L., *J. Exp. Med.*, 1914, xix, 195.

occasional *Macacus rhesus* monkey. The method was to inject intracerebrally an emulsion of the virus, in order to secure infection and by that means recent material for glycerolation. When the infection seemed to follow more readily than ordinarily, a Berkefeld filtrate was prepared and injected in the same manner, in order to determine whether any rise in potency had occurred.

No appreciable difference in the readiness with which infection could be induced or in the severity of the disease was observed until February of the year 1918, when it was found that not only was the emulsion effective, but also the Berkefeld filtrate prepared from it. The next step was to employ diminishing quantities of the filtrate in order to ascertain the degree of activity of the virus. In the tests which follow, this degree has been determined up to the point of establishing a large increase in potency.

EXPERIMENTAL.

Monkey 1.—Jan. 28, 1918. *Macacus rhesus* given intracerebrally 2 cc. of a Berkefeld filtrate of a 5 per cent glycerolated suspension of spinal cord and brain.[3] Feb. 4. Ataxia; movements slow; facial asymmetry (paralysis). Feb. 5. Died. The autopsy disclosed well marked lesions visible to the naked eye in the medulla and the spinal cord. The microscope revealed the typical conditions of experimental poliomyelitis in the midbrain, spinal cord, and intervertebral ganglia.

Monkey 2.—Apr. 3, 1918. *Macacus rhesus* given intracerebrally 0.5 cc. of a Berkefeld filtrate of a 5 per cent suspension of the glycerolated spinal cord and brain of the previous animal. Apr. 10. Ataxia, tremor of head; paralysis of arms, back, and neck. Apr. 11. Prostrate. Apr. 15. Died. The midbrain, spinal cord, and intervertebral ganglia showed typical lesions of experimental poliomyelitis.

This particular strain of the M. A. virus was now passed from time to time through other monkeys, and the restored activity was found to endure. After a further resting period, suspensions of glycerolated materials from Monkeys 1 and 2 were passed through two monkeys in September and October, 1921. The animals developed typical paralytic effects and were etherized on the 10th and 12th days respectively after the intracerebral inoculations.

With the active brain and cord material derived from these animals, a new series was inoculated into Monkeys 5, 6, 7, and 8.

Monkey 5.—Nov. 3, 1921. *Macacus rhesus* received 0.2 cc. of a 5 per cent glycerolated suspension intracerebrally. Nov. 8. Excitement. Nov. 9. Excitement increased; tremor of head. Nov. 11. Ataxia; left facial paralysis; deltoids weak. Nov. 13. Legs weak; convulsions. This animal was nursed to recovery; a residue of paralysis remained.

[3] The inoculations were made on etherized animals.

Monkey 6.—Nov. 10, 1921. *Macacus rhesus* given 0.2 cc. of a Berkefeld filtrate intracerebrally. Nov. 16. Excitement. Nov. 17. Tremor of head, and paralysis of face and both arms. Nov. 18. Left leg paralyzed. Etherized. The macro- and microscopic lesions of the central nervous organs are typical of experimental poliomyelitis.

Monkey 7.—Nov. 18, 1921. *Macacus rhesus*, 0.02 cc. of a Berkefeld filtrate of a 5 per cent suspension injected intracerebrally. Nov. 24. Excitement. Nov. 26. Tremor of head, ataxia, and paralysis of arms and legs. This animal was nursed to recovery. The left arm remained paralyzed; the other effects disappeared.

Monkey 8.—Nov. 28, 1921. *Macacus rhesus*, 0.02 cc. of a Berkefeld filtrate of 5 per cent suspension. Dec. 5. Excitement; head tremor; convulsions. Dec. 6. All extremities and back paralyzed; almost continuous convulsions. Etherized. The lesions present in the central nervous organs were typical.

The foregoing protocols show conclusively that the M. A. strain of virus which had become greatly reduced in potency in 1912, had regained a large part if not all of the lost power by 1918, and the revived virus was still potent in 1921.

In 1922 another test was made of the restored virus of 1918.

A portion of the glycerolated brain and cord tissue of Monkey 2 was emulsified and suspended in salt solution in strength of 5 per cent. On Feb. 1, a *Macacus rhesus* was injected intracerebrally with 0.5 cc. of this suspension. By Feb. 8, widespread paralyses had developed and the animal was etherized. A Berkefeld filtrate was prepared from a 5 per cent suspension of the brain and spinal cord of this animal, of which 0.2 cc. was injected intracerebrally into another *Macacus rhesus* on Feb. 9. Complete paralysis of the extremities and back and a left facial palsy were present on the 16th. Although an effort was made to save the animal with nursing, it was unsuccessful. Death occurred on Feb. 18. Typical lesions of experimental poliomyelitis were present in the central nervous organs.

This experiment showed, therefore, that the restored virus of 1918 was still highly active after a resting period of about 4 years in glycerol.

SUMMARY.

A strain of the virus of poliomyelitis has been described which has passed through several stages of virulence as tested upon monkeys.

The first stage consisted of the adaptation of the original human virus to the monkey. In this process high virulence was readily achieved.

The adapted, virulent strain of virus was passed regularly through monkeys and maintained its activity for about 3 years, when diminution became apparent. The loss of power of the virus was such that it may be said to have returned approximately to the level of the original human virus. This change constituted the second stage.

The third stage is represented by recovery of the high virulence. This revival occurred, it seems, during the sojourn of the virus in glycerol and required several years for its consummation. It was first noticed nearly 6 years after the low level of the second stage became established. The potent virus of the third stage has been found to remain active over a period of at least 4 years while preserved in glycerol.

What constitutes at least a superficial resemblance between the wave-like rises and falls of the incidence of epidemic poliomyelitis and the phenomena of increase and decrease in virulence of the specimen of virus has been alluded to. The two processes differ, however, essentially in respect to the time factor, since the fluctuations of the epidemic wave occupy small and those of the virulence occupy large increments of time.

STUDIES ON PNEUMOCOCCUS GROWTH INHIBITION.

I. The Protective Action of Gelatin for Pneumococci in Suspension.

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(Received for publication, September 18, 1923.)

During the course of certain experiments on the inhibition of the growth of pneumococci in a serum-leucocyte mixture, which are presented in detail in the second paper of these studies, there occurred certain irregularities in growth that seemed to be traceable to variations in the pneumococcus suspension used. At times growth failed to take place in the control tubes where it should have occurred had the organisms been present in the calculated numbers or in a suitable condition at the time of seeding. The method of making up the original suspension appeared to preclude much variation in numbers, and great care was taken to use organisms during their active growth phase. The probability was, therefore, that the solution used for suspension had an injurious effect on the organisms.

In these experiments Locke's solution was employed instead of the customary normal salt solution, because of Shearer's (1) findings that pneumococci (in common with several other organisms studied) when suspended in 0.85 per cent NaCl solution, showed evidence of early injury which resulted in the death of the organisms after a few hours. If, on the other hand, a balanced Ca-Na solution[1] was used as the suspension fluid, the organisms could be maintained in an apparently uninjured state for relatively long periods of time. Shearer, however, worked with large numbers of bacteria, while in the present experiments minute quantities were employed—0.0000001 cc. of a suspension containing approximately 1,000 million pneumo-

[1] Shearer used Ringer's solution.

cocci per cc. The use of such small numbers of organisms constitutes a much more severe test of the suitability of the suspension fluid. The death of a few hundred or thousand organisms in a heavy suspension would make no appreciable difference in its viability, while the death of a small number in a suspension containing only 50 or 100 organisms per unit of solution might result in a sterile suspension.

The Locke's solution employed was adjusted to pH 7.8 to 8.0, since it was assumed that the hydrogen ion concentration found to be most favorable for the growth of pneumococci (Dernby and Avery (2)) was also the most suitable for suspension in a non-nutrient fluid. However, in view of the irregularities in growth cited above, it seemed important to investigate the effect of varying the reaction of the Locke's solution. Furthermore, the possibility existed that the presence of a small quantity of protein in Locke's solution, or in other crystalloid solutions, might add something to their protective value. Accordingly, the following experiments were undertaken, with the purpose of determining the type of non-nutrient fluid most suitable for the suspension of pneumococci with a minimum amount of injury.

Standardization of the Pneumococcus Suspension.

1. Number of Organisms.—In order to obtain comparative results with the different solutions used, it was necessary to add the organisms in constant quantities. For the purpose of standardizing the suspension of pneumococci, Gates' (3) method of measuring the opacity of bacterial suspensions was employed. By means of bacterial counts it was determined that a suspension containing approximately 1,000 million pairs of pneumococci gave a corrected reading (Gates) of 2.1. In the following experiments the standard suspension of pneumococci used was always one with an opacity represented by the corrected reading 2.1.

2. Culture Media.—The culture medium used for growing the organisms for the suspension was meat infusion broth having a pH of 8.0. In order to obtain a vigorously growing culture it was found necessary to enrich the medium by adding 0.05 to 0.1 per cent dextrose[2] and 2 to 3 per cent rabbit serum.

3. Growth Phase.—Suspensions were always made from organisms in the active growth phase. To prepare the culture for the test, three tubes of serum broth (5 cc.), to which 0.05 per cent dextrose had been added, were inoculated with a loopful, 0.05 and 0.01 cc., respectively, of the stock culture (blood broth) and allowed to incubate overnight, usually for 14 to 15 hours. The tube showing the most active growth was used for subculturing. 1 to 5 cc. of culture, the amount

[2] The broth medium made with meat obtained in Peking is poor in sugar.

depending on the time at which it was desired to make up the suspension, were transplanted into 25 cc. of the serum-dextrose (0.1 per cent) broth contained in a 50 cc. centrifuge tube. If the broth has been kept in the ice box it is advantageous to take off the chill before inoculation with culture. Growth was allowed to proceed to the point at which transparency of the contents had almost disappeared. The tube was then centrifuged at high speed for 20 minutes and the supernatant fluid removed. The sediment was taken up in 4 to 5 cc. of the solution to be used for suspension and after thorough mixing, opacity readings were made and the standard suspension prepared.

4. Dilutions.—To make up the dilutions of the pneumococcus suspension, 1.8 cc. of the diluting fluid was placed in each of six test-tubes (15 mm. × 15 cm.). 0.2 cc. of the standard suspension, well mixed previously, was then added to the first tube and after thorough mixing, 0.2 cc. from each tube was successively added to the next, thereby making a series of dilutions ranging from 0.01 to 0.0000001 cc. of the standard, per 0.1 cc. of the dilution, or, in numbers, from approximately 10 million to 100 organisms. In order to make the dilutions as accurate as possible the following technique was employed. The same pipette was always used through the procedure. Fluid from the standard suspension was drawn up to the 0.7 mark on the pipette or slightly above this level, care being taken not to raise up the column of fluid higher than the 0.6 mark. 0.2 cc. was then delivered into the first tube from the 0.7 to the 0.9 marks and the remainder returned to the standard suspension tube. Thorough mixing of the tube's contents was obtained by tapping the bottom of the tube against the palm of the hand with a rotary motion. The first three tubes of each dilution were shaken for 3 minutes, and the last three for 4 minutes each, the pipette meanwhile being held in the mouth near the upper end. Before using the pipette for the next dilution it was rapidly passed through the flame once or twice, with a rotary motion. In order to prevent carrying over to the second dilution some of the standard suspension which might still be adhering to the inner wall of the pipette, the first dilution (before transfer) was drawn up several times to a point —the 0.5 mark or thereabouts—well above the level to which fluid from the previous concentration had reached. This process was repeated before each successive transfer—*i.e.*, each time the pipette was washed to a higher level than the time before—but the 0.2 cc. was always delivered from the 0.7 to the 0.9 marks. The dilutions were not made up until just before they were needed for the test. This precaution was probably not necessary, since it has been found that suspension in a suitable fluid, for several hours at least, probably does not injure the pneumococcus.

5. Organism Used.—The strain of organisms used in these experiments was a Type I pneumococcus, isolated 4 years ago from a case of lobar pneumonia, kept since in blood broth. Just before the beginning of these tests it was passed rapidly through four guinea pigs. It grew readily in artificial media; 0.0000001 cc. of the standard suspension, diluted in a suitable suspension fluid, and planted in dextrose blood broth, gave an abundant macroscopic growth in 12 hours. The organism was highly virulent for rabbits and mice.

Preparation of Solutions.

1. Locke's Solution.—The Locke's solution was made up as follows:

$NaCl$	0.9	gm.
$CaCl_2$	0.024	"
KCl	0.042	"
$NaHCO_3$	0.01 to 0.05	" *
H_2O	100.00	cc.

* Depending on the pH desired.

2. Salt Solution.—Where the per cent of NaCl is not mentioned, 0.9 per cent is indicated.

3. Gelatin Solutions.—These solutions were made by adding 0.1 per cent gelatin ("Difco bacto") before sterilization. The gelatin is most easily dissolved in distilled water heated gently and stirred during the process.

4. Water.—The water employed for making the solutions had been distilled twice in a glass still and boiled immediately before using, in order to render it CO_2-free.

5. Adjustment of Reaction.—On account of the marked changes in reaction that an adjusted solution always undergoes during sterilization, the solutions for the most part were not adjusted before autoclaving. At the beginning of the work the desired reaction was obtained by adding varying amounts of $NaHCO_3$ (from 0.2 to 1.0 cc. of a 0.5 per cent solution) before sterilization, to a series of 50 cc. quantities of the freshly prepared solution contained in small flasks. The reactions of these several flasks after autoclaving were usually found to lie between pH 7.0 and 8.0 with probably two or three of them at the H ion concentration desired. While this method obviated the necessity of adjustment after sterilization, it was uncertain and involved making up a great deal more of the solution than could be used.

Later it was found much simpler to sterilize the solution without adding $NaHCO_3$ and let the flasks stand in the ice box for several days until the exchange of CO_2 had come to an equilibrium, before adjusting. One of the flasks was then adjusted without using the gas flame, which introduces an increased concentration of CO_2 and thereby causes a change in the solution toward acidity.[3] The amount of N/10 $NaHCO_3$ found necessary to bring the contents of this flask to the desired pH was next added to each of the other flasks. These latter flasks were of course flamed on opening, in order to preserve sterility. While there is an immediate change in reaction following flaming, solutions containing bicarbonate have been found to return, after several hours, to their former H ion level. In the latter part of the work the solutions, with the exception of Locke's, were buffered after adjustment with M/15 balanced phosphate mixture (sterilized by autoclaving). The buffer was added in the ratio of 1 part of buffer to 50 or 100 parts of solution. This increased considerably the stability of the reaction.

[3] Factors influencing the degree of change in reaction due to flaming will be given in detail in a subsequent communication.

Culture Media Employed.

In testing the viability of the organisms in suspension, dextrose blood broth was used chiefly. To meat infusion broth, adjusted, with a pH of 8.0 and buffered with 0.1 per cent K_2HPO_4, 2 per cent defibrinated blood and 1 per cent dextrose were added. The broth was then distributed into test-tubes in 5 cc. quantities and incubated for 24 hours before use. A single lot of dextrose blood broth was employed in each experiment. For plating, 1 per cent dextrose rabbit blood agar, of pH 7.8 to 8.0, was used.

The technique of plating was carried out as follows: First, 1 cc. of defibrinated rabbit blood was delivered into a deep plate. Next, 0.1 cc. of the suspension to be cultured was placed in the plate near the blood, with which it was immediately mixed. Finally, 10 to 12 cc. of agar, at a temperature of 40–41°C., which had been cooled at 43° for at least 15 minutes previously, were poured into the plate and the contents well mixed by rotation. By this means a greater number of colonies was obtained than when the culture material was added to the agar in the tube, as is usually done.

The inoculated tubes and plates were always observed at frequent intervals for at least 72 hours before being considered negative.

Suspension in Locke's and Gelatin-Locke's Solutions.

Experiment 1.—(Table I.) The standard suspension was made up in gelatin-Locke's solution. From this four separate series of dilutions were made, two in Locke's and two in gelatin-Locke's solution. Immediately after the completion of the final dilution 0.1 cc. of this suspension was plated in rabbit blood agar. Two of the suspensions, one Locke's and one gelatin-Locke's, were left at room temperature; the other two were placed in the incubator. Cultures from these tubes (0.1 cc. of the suspension) after thorough agitation, were made into dextrose blood broth, pH 8.0, at short intervals of time for a period of 30 hours, as indicated in the table.

It is seen in Table I that the pneumococci suspended in gelatin-Locke's solution remained viable for a very much longer time than did those in the plain Locke's solution. How long the pneumococci would have survived in gelatin-Locke's solution at room temperature is not known, since the test was terminated at the end of 30 hours. The fact that the gelatin-Locke's suspension kept at incubator temperature yielded sterile cultures after 9 hours indicates that there could have been little if any growth in this solution.

Experiment 2.—(Table II.) The standard suspension of pneumococci was made up in gelatin-Locke's solution, pH 7.6. From this suspension dilutions

were made into gelatin-Locke's solutions ranging in pH from 7.0 to 8.0. Each dilution was shaken for only ½ to 1 minute instead of the customary 3 to 4 minutes. Cultures of the test suspensions were made as in Experiment 1, using the same test media.

TABLE I.

Survival of Pneumococci in Locke's and Gelatin-Locke's Solutions at Room and Incubator Temperatures.

Solution used.	No. of organisms.		Culture in dextrose blood broth.							
	Amount of standard suspension.	Colonies in plate.	At hrs.							
			0	3	6	9	12	19	24	30
Room temperature.	*cc.*									
Locke's pH 7.7.	0.000001		+	+	+	+	0	0	0	
" pH 7.7.	0.0000001	46	+	+	+	+	0	0	0	
Gel.-Locke's pH 7.8.	0.000001		+	+	+	+	+	+	+	+
" pH 7.8.	0.0000001	40	+	+	+	+	+	+	+	+
Incubator temperature.										
Locke's pH 7.7.	0.000001		+	0	0	0	0	0	0	
" pH 7.7.	0.0000001	38	+	0	0	0	0	0	0	
Gel.-Locke's pH 7.8.	0.000001		+	+	+	+	0	0	0	0
" pH 7.8.	0.0000001	45	+	+	+	0	0	0	0	0

TABLE II.

Survival of Pneumococci in Gelatin-Locke's Solutions of Varying Hydrogen Ion Concentration.

pH of solution.	Amount of standard suspension.	Culture in dextrose blood broth.					
		At hrs.					
		4	9	15	18	21	24
Room temperature 24–25°C.	*cc.*						
8.0	0.0000001	+	+	+	+	+	+
7.8	"	+	0	0	0	0	0
7.6	"	+	+	+	+	+	+
7.5	"	+	0	0	0	0	0
7.3	"	+	+	+	+	+	+
7.2	"	+	+	+	+	+	+
7.0	"	+	+	+	+	+	0
Incubator temperature 37°C.							
7.6	0.0000001	+	+	0	0	0	0

The results of this experiment show that the reaction of gelatin-Locke's solution may be varied over a range of pH 7.0 to 8.0 without affecting appreciably its protective properties for pneumococci. The early death in the suspensions of pH 7.8 and 7.5 could be interpreted in only one way; namely, that these two dilutions contained much fewer organisms than the others, as a result of inadequate mixture during the process of making the dilutions. Further experiments showed this assumption to be correct. It was found that when the time of agitation was shortened much below that of the standard adopted, marked irregularities in the final dilution resulted. With dilutions properly prepared, a seeding into plates of 0.0000001 cc. of the standard suspension (0.1 cc. of the sixth dilution) made in gelatin-Locke's solution, showed on the average 40 to 60 colonies. Sometimes the number was a little below or somewhat above this range.

Suspension in Salt Solution and Water with and without Gelatin.

In order to determine whether the addition of gelatin to fluids other than Locke's would likewise confer on them the same increased protective action for pneumococci shown by gelatin-Locke's solution, the following experiments were undertaken.

Experiment 3.—(Table III.) The solutions with the exception of gelatin-Locke's, were buffered with 1:100 phosphate mixture (pH 7.8). The standard suspension and the main series of dilutions were made up in gelatin-water. In order to save time the dilutions in plain water were begun from the third dilution of the gelatin-water series, *i.e.* 0.2 cc. of the third gelatin-water dilution was placed in 1.8 cc. of plain water, and the remaining three dilutions continued in this fluid. Similarly the dilutions in plain salt solution, 0.9 per cent, were begun from the third gelatin-water dilution. The other gelatin solution suspensions were begun from the fourth dilutions of the gelatin-water series. The sixth dilution in each case was made up to double the amount of the preceding dilutions in order to provide sufficient fluid for testing H ion concentration. In each case, immediately after the completion of the final dilution, 0.1 cc. of this suspension was plated in dextrose blood agar. The suspensions were then kept at a temperature of 23°C. except for the first 5 or 6 hours during which time the initial temperature of 26° dropped gradually to 23°C. At successive intervals of time, 0.1 cc. of the contents of the suspension tubes, after 3 to 4 minutes' agitation, was transplanted into dextrose blood broth. The reaction of the several fluids was determined immediately after distribution into the dilution tubes and at the end of 24 hours the H ion concentration of each suspension tube's contents was tested.

TABLE III.

Comparison of the Survival of Pneumococci in Salt Solutions and Water with and without Gelatin.

Solution used.	pH of solution at beginning.	Temperature at which suspension was maintained.	No. of colonies in plate.	Culture in dextrose blood broth. At hrs. 0	1	3	6	9	20	24	48	72	96	120	144	168	192	pH of solution at 24 hrs.
		°C.																
NaCl 0.9 per cent.	7.8	23	0	0	0	0	0	0	0	0								8.0
Gel.-NaCl 0.9 per cent.	7.8	23	41	−	+	+	+	+	+	+	+	0	0	0	0			7.8
Water.	7.8	23	43	+	0	0	0	0	0	0								8.1
Gel.-water.	7.8	23	48	−	+	+	+	+	+	+	+	+	+	+	+	+	0	7.8
Gel.-Locke's.	7.7	23	45	−	+	+	+	+	+	+	+	+	+	+	+	0	0	7.9
NaCl 0.9 per cent.	7.8	37	0	0	0	0	0	0	0	0								8.3
Gel.-NaCl 0.9 per cent.	7.8	37	34	−	+	+	0	0	0	0								8.2
Water.	7.8	37	50	+	0	0	0	0	0	0								8.4
Gel.-water.	7.8	37	49	−	+	+	+	+	0	0								8.0

The contrast between the gelatin and non-gelatin solutions is strikingly brought out in Table III. No organisms could be recovered from the plain salt solutions at the completion of the dilutions, while suspensions in gelatin-salt 0.9 per cent solution remained alive for 48 hours. Similarly with water, pneumococci could not be recovered from the water suspension after 1 hour's standing, while those organisms suspended in gelatin-water remained viable for 7 days. It will also be noted that in gelatin-water and gelatin-Locke's solutions pneumococci remained alive three times as long as they did in gelatin-salt solution.

The fact that the reaction of the water suspension at 23°C. was found to be at pH 8.1 after 24 hours, suggested the possibility that the difference shown by this experiment between water and gelatin-water might, at least to a certain degree, be referable to the greater alkalinity of the water. However, a repetition of this portion of the experiment, in which the reaction of the solutions remained between the limits of pH 7.4 to 7.9, gave identical results. A further experiment was performed, involving the use of large test-tubes for making the final dilution, so that 10 cc. quantities of fluid could be used. In this way it was possible to test the reaction in the suspension tube repeatedly during the course of the experiment. The reaction remained constantly at pH 7.6 to 7.7 and the results agreed with those of the two preceding tests. 0.5 per cent NaCl solution was found to be equally unsuitable as a suspension fluid for pneumococci.

The presence of phosphate was found to have no other than a beneficial effect. Further experiments showed that twice the quantity used in Experiment 3, *i.e.* 1:50, could be used without injury to the pneumococci.

Nature of the Protective Action of Gelatin-Containing Solutions.

In attempting to account for the manner in which the presence of 0.1 per cent gelatin in Locke's solution, salt solution, and water adds so greatly to their protective value as suspension fluids for pneumococci, several possible explanations presented themselves. First to be considered was the possibility that even at room temperature slow growth might occur in a gelatin solution of this concentration. While the presence of growth could hardly account for the difference

found between gelatin-containing and non-gelatin-containing solutions immediately at the completion of the diluting process, yet it might well explain the prolonged life of pneumococci in the former. Accordingly series of plates were made at short intervals of time for periods of 24 to 96 hours on suspensions of pneumococci kept at 22–26°C. and also at 37°C. The results of these tests gave no definite evidence of growth either at incubator or room temperature. There was a progressive diminution in the number of colonies in successive plates, which became marked after 18 to 24 hours. Similar tests were made on suspensions of pneumococci in gelatin solutions of higher gelatin concentrations kept at 37°C. It was not until 0.5 per cent gelatin had been reached that definite evidence of growth was obtained.

Next it was considered possible that agitation of pneumococci in solutions of crystalloids or water might result in injury to the organism and that the action of gelatin might be to protect against such mechanical injury. For Rous and Turner (4) have found that gelatin solutions protect the red blood cells against injury during manipulation.

Experiment 4.—(Table IV.) For this experiment the solutions were buffered with phosphate 1:50 at pH 7.8. A standard suspension was made in gelatin-water and two series of dilutions in water, one in tubes as usual and one in 25 cc. flasks, each of which received 4.5 cc. of solution. At the completion of each flask dilution 0.5 cc. was transferred to the next flask. The tubes of water and gelatin-water were agitated together for the customary lengths of time but rather more vigorously than usual. The flasks were rotated gently, first in one direction and then in the other, for similar lengths of time. Then in order to prevent further injury from agitation the final dilutions of the water series were distributed in 0.1 cc. amounts into a number of small tubes, 1 × 12 cm., each containing 0.9 cc. water, adjusted and buffered as above. This quantity of fluid was sufficient to reduce to a minimum any disturbing effect of evaporation. The sixth dilution of gelatin-water was also distributed in like amounts into tubes containing gelatin-water. Two plates were made at this time from each of the three suspensions. The tubes were kept at a constant temperature of 22–23°C. At varying intervals of time, as indicated in the table, 5 cc. of dextrose blood broth were transferred to one tube of each series, which was then incubated. By this method of culture, any loss of pneumococci which might result from transfer of part of the suspension into the broth, was avoided. Into four tubes of each series, 2 cc. amounts of the water or gelatin-water used above were delivered. These tubes were used for the determination of H ion concentration at intervals during the course of the experiment.

Table IV shows that pneumococci may be so injured by agitation in water that no organisms are recoverable from the suspension by the time the last dilution is completed. The series of dilutions in water carried out by rotating flasks gently exhibited much less injury of the organisms, relatively speaking, as shown by the fact that they could be recovered from suspensions as long as 12 hours afterwards. The contrast, however, between gelatin-water and water (both agitated to the same degree) is striking. The organisms suspended in gelatin-water were alive at the end of 30 hours, and, judging from previous experiments, they would continue to live for several days to a week. The fact that the reaction of the water at 20 hours was pH 8.2, while that of the gelatin-water was pH 8.0, probably had no effect on the

TABLE IV.

Protective Action of Gelatin against Mechanical Injury.

Solution used.	Method employed in making dilutions.	No. of colonies in plate.	Culture in dextrose blood broth. At hrs.								pH of solution. At hrs.			
			0	1	3	6	9	12	20	24	30	0	5	20	30
Water.	Agitation in tube.	0	0	0	0	0	0	0	0	0		7.5	7.7	8.2	8.0
"	Rotation in flask.	16	+	+	+	0	0	+	0	0		7.5	7.7	8.2	8.0
Gel.-water.	Agitation in tube.	53	+	+	+	+	+	+	+	+	+	7.4	7.5	8.0	7.9

outcome of the experiment, since it has been found that pneumococci survive well in suspension fluids with a reaction as alkaline as pH 8.2. An H ion concentration, however, of pH 8.3 to 8.4 seems to be definitely unfavorable. In a repetition of the experiment, in which the reaction remained constant at pH 7.9 to 8.0 in both the flask and tube dilutions, organisms were recovered from the tube only immediately at the completion of the dilutions, in broth but not in plate, while three plates from the flask gave an average of 51 colonies (34, 52, 68, respectively) and positive broth cultures were obtained from the suspension for 3 hours. The degree of protection afforded by the presence of gelatin in water seems to be very great, since even the most vigorous shaking that could be given by striking the tube against the hand did not apparently injure the pneumococci.

From the results of the above experiment, which was repeated several times, it seemed not improbable that the whole effect of gelatin was to protect against mechanical injury; and that in the absence of agitation, solutions without gelatin might prove as suitable suspension fluids as those containing gelatin, with the possible exception of salt solutions. In order to test this possibility an experiment was devised whereby a suspension containing small numbers of pneumococci prepared with as little injury as possible, could be delivered into a series of solutions with and without gelatin, for comparison of time of viability.

TABLE V.

Preservative Action of Gelatin Solutions.

Solution used.	Per cent of gelatin in inoculum fluid.	Final per cent of gelatin in suspension fluid.	Growth in dextrose blood broth. At hrs.									pH of solution. At hrs.			
			0	1	3	6	9	12	20	24	30	0	5	20	30
	per cent	*per cent*													
Water.	0.001	0.0001	+	+	+	+	+	0	0	+	0	7.5	7.7	8.2	8.0
Gel.-water.	"	0.1	+	+	+	+	+	+	+	+	+	7.4	7.5	8.0	7.9
Salt sol.	"	0.0001	+	+	+	0	0	0	0	0	0	7.6	7.9	8.2	8.2
Gel.-salt.	"	0.1	+	+	+	+	+	+	+	+	0	7.7	7.9	8.2	8.2
Locke's sol.	"	0.0001	+	+	+	+	+	0	0	0	0	7.1	7.6	7.7	7.7
Gel.-Locke's.	"	0.1	+	+	+	+	+	+	0	0	0	7.3	7.5	8.0	8.0
Water.	0.01	0.001	+	+	+	+	+	+	0	0	0	7.5	7.7	8.2	8.0
Gel.-water.	"	0.1	+	+	+	+	+	+	+	+	+	7.4	7.5	8.0	7.9
Salt sol.	"	0.001	+	+	+	+	+	0	0	0	0	7.6	7.9	8.2	8.2
Gel.-salt.	"	0.1	+	+	+	+	+	+	+	+	+	7.7	7.9	8.2	8.2
Locke's sol.	"	0.001	+	+	+	+	+	0	0	0	0	7.1	7.6	7.7	7.7
Gel.-Locke's.	"	0.1	+	+	+	+	+	+	0	+	+	7.3	7.5	8.0	8.0

Experiment 5.—(Table V.) Solutions prepared as in Experiment 4. Standard solution and main series of dilutions made in gelatin-water. In order to diminish as much as possible the amount of gelatin in the final dilution to be used for the inoculation, and at the same time to reduce to a minimum the injury which the pneumococci undergo when agitated in plain water, 0.1 cc. of the fourth dilution in the gelatin-water series was transferred into 9.9 cc. of plain water contained in large test-tubes 25 mm. × 20 cm. In this way the sixth dilution was reached with only one period of agitation in water and this contained 0.001 per cent of gelatin. As a control, a second dilution in plain water was made from the fifth dilution of the gelatin water series in the usual way, since it was feared that a

single agitation in water containing as little gelatin as 0.001 per cent might cause injury to the organisms. These two suspensions were delivered in 0.1 cc. quantities into a number of small tubes 1 × 12 cm. each containing 0.9 cc. of the solution to be tested. Half way through this process five plates were made from one of the suspensions and four from the other. Successive tenths of a cc. from the 0.1 to 0.9 marks on the pipette were used. The same pipette was used throughout with each suspension. Also into four tubes of each series 2 cc. of the solution were delivered for purposes of testing the H ion concentration at intervals during the test. The tubes were kept at a temperature which remained constantly between 22 and 23°C. At successive periods of time, indicated in the table, 5 cc. dextrose blood broth were transferred into one tube of each series, which was then incubated.

Table V shows that in every instance the pneumococci survived for a longer period of time in the gelatin-containing solutions than in those containing no gelatin. This difference was most marked in the case of salt solution. The number of organisms in the inoculum was about that employed in the previous experiments; the nine plates showed from 38 to 62 colonies—an average of 52. A repetition of a part of the experiment with water, gelatin-water, salt solution, and gelatin-salt, gave essentially the same results. In this latter experiment the test was run for a longer period, and the difference in the survival time between water and gelatin-water was considerably greater. No organisms could be recovered from the water after 6 hours, while in the gelatin-water, organisms were still found to be viable at 72 hours. (The H ion concentration of the solutions remained more constant—between pH 7.4 and 7.9 throughout the entire test.) Since the organisms implanted in the various test solutions were in the same state and approximately in the same numbers to begin with, and the factor of mechanical injury as a variant was entirely excluded, it seems fair to conclude that the longer period of survival in the gelatin-containing solutions indicates a definite preservative or maintaining action of gelatin for pneumococcus, in addition to the protection it affords against mechanical injury. The term preservative is used simply to indicate a second distinct property of the gelatin solutions, which should be properly included under the general designation of protective action, since the prolonged life of the organisms indicates protection against those influences causing early death in the non-gelatin-containing solutions.

It should further be pointed out that in the first part of the test in which the inoculum fluid contained a very small amount of gelatin, the pneumococci lived a much shorter length of time in salt solution than in either Locke's solution or water. This has been found in subsequent tests, and corroborates Shearer's findings regarding the toxicity of NaCl solutions for pneumococci.

The Beneficial Effect of Phosphate in Gelatin-Salt 0.9 Per Cent Solution.

While the results of the foregoing experiments seemed to demonstrate that the addition of 0.1 per cent gelatin to 0.9 per cent NaCl solution produces a suitable fluid for suspension of pneumococci, attempts to utilize this solution as the medium for suspension and dilution in certain experiments on growth inhibition, showed clearly that the organisms were markedly injured during their sojourn in the gelatin-salt solution. A resumé of the protocols of the tests on gelatin-salt showed that in each instance in which gelatin-salt 0.9 per cent was used, the main series of dilutions had been made up in either gelatin-salt 0.5 per cent or gelatin-water, and that the dilutions in gelatin-salt 0.9 per cent were made from the fourth or fifth dilutions of this main series. Furthermore, in every experiment in which gelatin-salt 0.9 per cent showed well marked protective properties for pneumococci, phosphate was present in the solution. The gelatin-salt solution used for the pneumococcus suspension in the above mentioned experiments on growth inhibition did not contain phosphate. It seemed probable, then, that either the greater length of time of agitation in gelatin-salt 0.9 per cent solution or the absence of phosphate would account for the lack of protection shown. In the following experiment the effect of each of these two factors was tested.

Experiment 6.—(Table VI.) The standard suspension was made up in gelatin-salt 0.5 per cent, and one series of dilutions made in this same fluid. The gelatin-salt 0.9 per cent series, with and without phosphate, were each begun from the standard suspension. The phosphate-containing solutions were adjusted and buffered by first adding 1:50 H_3PO_4 then NaOH to the desired reaction.

One series of dilutions was made in gelatin-water (containing phosphate), from the fourth dilution of which the series was continued in gelatin-salt 0.9 per cent (indicated in the table). The sixth dilution of the above suspensions was made in 10 cc. quantities (in 50 cc. test-tubes) and kept at a temperature of

20–22°C. At intervals, the tubes were agitated 3 to 4 minutes, and 0.1 cc. of the contents transplanted into 5 cc. dextrose blood broth. Also 2 cc. quantities of the suspension were removed from time to time for testing the H ion concentration. The last two gelatin-salt, 0.9 per cent suspensions shown in the table were put up at a different time, and from another gelatin-water series of dilutions. The suspension was distributed into small test-tubes, into which dextrose blood broth was transferred at intervals, as previously described.

TABLE VI.

Beneficial Effect of Phosphate in Gelatin-Salt 0.9 Per Cent Solution.

Solution used.	Amount of phosphate.	No. of colonies in plate.	Culture in dextrose blood broth. At hrs. 0	½	4	6	9	11	21	24	30	44	pH of suspension. At hrs. 0	2	½	21
	per cent															
Gel.-salt 0.5.	1:50	32	+	+	+	+	−	+	+	−	+	+	7.0	7.1	7.3	7.5
" " 0.9.	1:50	22	+	+	+	+	−	+	+	−	0	0	6.8–7.0	7.1	7.3	7.5
" " 0.9.	0	76	+	+	+	+	−	0	0	−	0	0	6.8–7.0	7.2	7.4	7.4
" " 0.9. From 4th dil. of gel.-water.	1:50	25	+	+	+	+	−	0*	+	−	0	0	7.2	7.3	7.4	7.5
Gel.-salt 0.9. From 5th dil. of gel.-water.	1:50		+	+	+	+	+	−	+	+	0	0	7.4†		7.5	7.6
Gel.-salt 0.9. From 5th dil. of gel.-water.	0		+	+	+	+	0	−	0	0	0	0	7.6†		7.7	7.9

* Culture tube contaminated.

† The pH of the final dilution tubes was 7.0 in the phosphate-containing, and 6.9 in the non-phosphate-containing suspensions.

The results of this experiment (Table VI) show clearly that the protective action of gelatin-salt 0.9 per cent depends very largely on the presence of the phosphate. In the absence of phosphate the pneumococci survived for only a brief period, regardless of whether agitation had been long or short. Additional experiments with varying concentrations of NaCl in gelatin-salt solution (containing phosphate) showed that in solutions of 0.6 per cent or 0.5 per cent NaCl, the survival time was definitely longer than when a higher percentage of NaCl was employed. In comparing gelatin-salt 0.9 per cent with gelatin-salt 0.5 per cent, both without phosphate, it

was found that pneumococci survived for a very much longer time in the latter solution—as long as 48 hours—but even gelatin-salt 0.5 per cent showed a definite increase in its protective properties after the addition of phosphate.

Certain other findings may be briefly summarized as follows: The protective action of gelatin for pneumococci suspended in non-nutrient fluids was found not to be peculiar to this substance alone. Serum added to salt solution, Locke's solution, or water in an amount such that the weight of the solids in the serum equalled 0.1 per cent of the solution, was found to exert a protective effect similar to that of gelatin. In attempting to account for the way in which gelatin protected pneumococci against the toxic action of NaCl, especially in 0.5 per cent solution without phosphate, the gelatin used was analyzed for its calcium content. This was found to be 38 mg. of Ca per 100 gm. of gelatin. The gelatin was now purified by the method of Loeb (5) and a reanalysis showed a calcium content of only 2.5 mg. per 100 gm. of gelatin. Pneumococci suspended in gelatin-salt 0.5 per cent made with this purified gelatin showed a survival period but slightly shorter than that which occurred in the non-purified gelatin-salt of the same per cent. Hence the beneficial effect of gelatin in salt solution of this concentration could not be accounted for, to but a small degree only, by the calcium content of the gelatin used.

DISCUSSION.

While the effect of shaking on certain other microorganisms has been studied, we have not been able to find any mention in the literature of the effect of agitation on the pneumococcus. Meltzer (6) observed many years ago that *Bacillus megaterium* could be injured by continuous shaking in both water and salt solution for a number of hours, but the effects were frequently not marked. *Bacillus subtilis* and a "yellow coccus from the air" were found by him to be even less susceptible to injury by shaking. Appel (7) repeated Meltzer's work and found that the bacteria could be injured only by violent agitation in tubes containing glass beads. Our results show that pneumococci, on the contrary, are markedly injured by a relatively brief period of moderately vigorous agitation when suspended in water or fluids containing only crystalloids.

The mechanism of the protective action of gelatin for pneumococci is not clear. Protection against mechanical injury may conceivably be due to the presence of the large gelatin molecules, acting to diminish the impact of collision between individual organisms and between organisms and the glass walls. It is possible that the gelatin may even form a protective coating around the individual pneumococci. Concerning the nature of the further protective effect of gelatin for pneumococci under static conditions or its preservative action, several possibilities present themselves. (1) The presence of gelatin in the solution may cause a retardation of autolysis by the enzymes of the bacterial cell. Lord and Nye (8) have put forth this theory to account for the longer life of pneumococci suspended in serum, as contrasted with similar suspensions in solutions of crystalloids. (2) Colloids such as gelatin may act to prevent penetration of the bacterial cell wall by the surrounding fluid—a process that, occurring in non-protein-containing solutions, may ultimately lead to death of the organism. (3) It is further possible that in protein-containing solutions pneumococci are enabled to carry on certain metabolic processes despite the absence of growth, which tend to prolong the life of the organism. (4) A very slow multiplication of the organisms, though unlikely, cannot be excluded, despite the results of the plating experiments which show after a certain number of hours a gradual and progressive diminution in pneumococci recovered from 0.1 per cent gelatin solutions kept at a temperature of 22–23°C.

The manner in which 0.1 per cent gelatin in 0.5 per cent NaCl solution protects the pneumococci against injury by the Na ion remains unexplained. The additional protective action of phosphate, however, in the higher concentrations of NaCl (in gel.-salt) suggests an antagonism between sodium phosphate and sodium chloride. In the last experiment described and others summarized there was no potassium present, since Na_2HPO_4 alone was used as buffer. While the study of antagonistic salt action has shown that the cations are chiefly concerned in this phenomenon, yet certain instances have been found in which anions played a definite rôle. Loeb (9) found that Na acetate was much more effective than NaCl in protecting the eggs of fundulus against the toxic action of KCl. Brooks (10) studying the action of various ions on the respiration of

Bacillus subtilis, showed that the depressant effect of NaCl could be neutralized by Na taurocholate. Further study is in progress to determine whether or not there exists a true antagonism between NaCl and Na_2HPO_4.

The presence of 0.1 per cent gelatin in the fluid used for the pneumococcus suspension, has been found not to interfere with phagocytosis or intracellular digestion of the pneumococci. Its effect on other immunological reactions has not been tested.

It seems not improbable that pathogenic microorganisms, as a class, are more susceptible to mechanical injury than is commonly supposed. In numerous bacteriological and immunological procedures, high dilution of the bacterial suspension used is required. In certain of these tests vigorous and often prolonged agitation is employed in making the dilutions. Even though sterility of the suspension is not produced by this process, the organisms may be so injured as to interfere seriously with growth. In view of the findings brought out by the study of pneumococcus suspensions, it may be found advantageous to employ gelatin more widely in solutions used for the suspension of bacteria.

SUMMARY.

Experiments were undertaken with the purpose of determining the type of non-nutrient fluid most suitable for the suspension of pneumococci with a minimum amount of injury. For comparative studies, dilutions in the various fluids tested were made from a standard suspension containing approximately 1,000 million pneumococci per cc. The protective properties of each solution were judged by the length of time viable organisms could be recovered (in culture) from a suspension containing 0.0000001 cc. of the standard suspension. The hydrogen ion concentration of the solutions, the temperature at which the suspensions were kept, and the culture media were carefully controlled. Furthermore, plates were made at the beginning of the experiment to determine the number of organisms present in a unit of suspension.

It was found that pneumococci suspended in Locke's solution, 0.5 and 0.9 per cent NaCl solutions, and water, remained alive for only

a few hours at most. Salt solution was shown to be the least suitable of these three; not infrequently by the time the 0.000001 cc. dilution in this fluid had been reached, the suspension was sterile. The addition, however, of 0.1 per cent gelatin to the above fluids transformed all, except 0.9 per cent NaCl, into highly favorable solutions. Pneumococci suspended in gelatin-water and gelatin-Locke's solution remained alive at room temperature for 6 to 7 days; in gelatin-salt 0.5 per cent solution, for at least 2 days. It was possible to vary the H ion concentration of the suspension fluids from pH 7.0 to 8.2 without any marked effect on the results. However, a pH of 7.4 to 8.0 appeared to be the most suitable.

The nature of the protective action of gelatin was investigated. Series of plates made at frequent intervals failed to reveal any growth in suspensions of pneumococci containing this small concentration of gelatin. The beneficial effect of gelatin in the above solutions was found to lie largely in its protection of the pneumococci against the mechanical injury which occurs during the process of dilution in crystalloid solutions or water. Gelatin shows in addition a well marked preservative action, that is to say, it protected organisms against early dissolution. The nature of this preservation is uncertain.

The presence of 0.1 per cent gelatin in 0.9 per cent NaCl failed to protect pneumococci against the toxic action of Na. But it was found possible to neutralize to a considerable degree this toxic effect of sodium by the addition of a small quantity (2 per cent) of a M/15 balanced phosphate mixture or sodium phosphate. In a gelatin-salt 0.9 per cent solution buffered with phosphate, pneumococci survived for 24 to 48 hours. Even in gelatin-salt, 0.5 per cent the presence of phosphate had a definite beneficial effect.

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STUDIES ON PNEUMOCOCCUS GROWTH INHIBITION.

II. A Method for Demonstrating the Growth-Inhibitory and Bactericidal Action of Normal Serum-Leucocyte Mixtures.

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Plate 20.

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The details of the phenomenon of immunity to pneumococci, natural and acquired, active and passive, are not yet entirely clear. The exact rôle which the blood plays in this phenomenon is not established. Certain facts, however, are known.

It has been generally held that the blood serum of naturally immune animals, such as the pigeon, does not confer immunity or protective properties on the susceptible animals. Bull and McKee (1), however, in 1921 reported experiments which indicate that the serum of normal chickens is capable of protecting mice and guinea pigs against infection with moderate sized doses of pneumococci. The blood serum of the animal which has been highly immunized artificially, however, is capable of conferring high grades of passive immunity on a susceptible animal. In this case the properties upon which resistance depends apparently reside largely in the blood serum.

Many attempts have been made to discover the mode of action of the serum of immunized animals in preventing or curing infection. It has been well established that the serum from the artificially immunized animal has certain properties not possessed by the serum of normal animals. It possesses the power of agglutinating pneumococci and of favoring phagocytosis; in other words, it has agglutinating and bacteriotropic powers. Its power of inducing agglutination has been thought by Bull (2) to play an important rôle in its protective action. Other observers think that the increased bacterio-

tropic action of the serum is of chief importance and that phagocytosis is the most important mechanism of which the immune animal makes use in guarding against infection. On the other hand, it has been impossible to demonstrate that immune serum or whole fresh blood of resistant animals possesses any bactericidal power or even power to inhibit the growth of pneumococci (Barber (3)).

In 1912, however, Wright (4) reported that when pneumococci are mixed with blood taken directly from the vessels of certain human individuals, large numbers of pneumococci are killed. According to him this phenomenon does not occur when the bacteria are mixed with whipped blood, or blood which is prevented from clotting by precipitating the calcium. He called this power the phagocytobactericidal power, thereby indicating that the leucocytes are an essential factor in this destructive action. The technique employed in this investigation is not accurately described but apparently the capillary pipette method was employed. It is obvious that in drawing any conclusions in regard to destructive action of serum or blood the effects of agglutination in causing an apparent decrease in numbers of bacteria must be taken into consideration.

In 1918 Heist and Solis-Cohen (5), employing a modified capillary pipette method, reported that when whole pigeon's blood before it coagulates is mixed with small numbers of pneumococci the bacteria fail to multiply. The failure to multiply was determined by blowing the clots out of the capillary tubes on to a slide and examining the stained specimen under the microscope. If instead of pigeon blood, rabbit or mouse blood was employed the bacteria grew with great vigor. This difference between the blood of immune and susceptible species as regards action on pneumococci was not seen if the blood was defibrinated before the bacteria were added. The writers considered the possibility that the difference between uncoagulated and defibrinated blood might depend on the fact that in the former the leucocytes and bacteria may remain distributed in the meshes of the clot and so phagocytosis be favored, but they think this fact would not explain the difference between the action of pigeon and rabbit blood.

Heist and Solis-Cohen (6) extended their studies with this method and found that the ability of a given strain to grow in rabbit blood was directly proportional to the virulence of the strain. Furthermore, they found that failure to grow in the blood of artificially immunized animals bore a direct relation to the degree of immunity possessed by the animals from which the blood was obtained. In blood taken from animals undergoing immunization to pneumococci the destructive action could be demonstrated before agglutinins appeared in the blood. It would seem, therefore, that the apparent failure to grow could not be related to agglutination.

Bull and Bartual (7), using a somewhat similar technique, showed that while the whole blood of immune animals is not so pneumococcidal as had been claimed,

nevertheless, when the blood of naturally immune animals is employed the latent period is prolonged as contrasted with the latent period when the blood of susceptible animals is used. Their observations showed, however, that artificial immunization did not cause the blood to acquire the property of prolonging the latent period of growth. They concluded that the retardation of multiplication which may occur depends upon the opsonization of the pneumococci and phagocytosis. The experiments did not indicate that the bacteria were actually killed since none of the cultures became sterile.

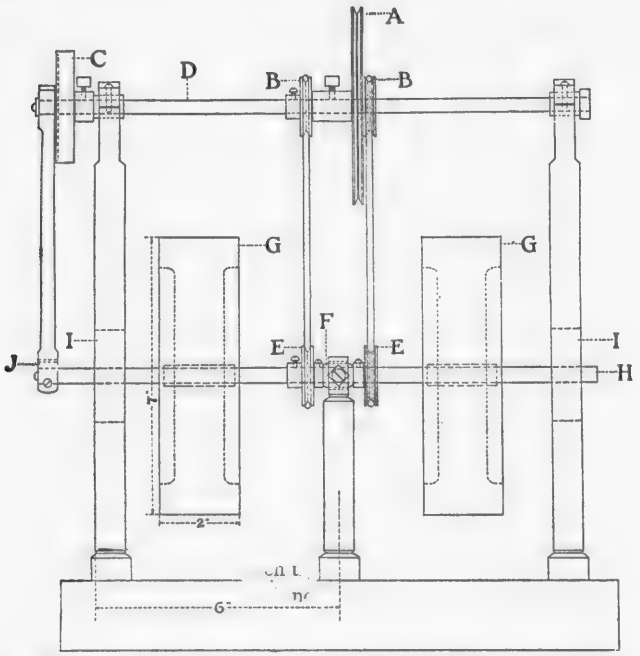
The experiments we have mentioned, therefore, do not certainly indicate whether the apparent retardation of growth in whole blood is simply the result of agglutination and phagocytosis or whether a new and previously unknown factor in immunity is operative.

We have considered the matter of sufficient importance to be investigated further and have attempted to modify the technique employed so as to imitate more closely the actual conditions present in the living animal. In the animal, fluid currents operate to bring the leucocytes and implanted microorganisms into intimate contact. In the capillary pipette, or test-tube, this constant mixing process is absent. Not impossibly, growth inhibition and death only occur if all the organisms come into contact with actively functioning leucocytes and are phagocyted by them. There is a strong probability that in any mixture of whole coagulated blood and pneumococci some of the organisms will escape phagocytosis, and hence, even though growth retardation occurs, the culture ultimately survives. Moreover, in the animal body the blood is not clotted and therefore the experiments carried out with whole blood are conducted under highly artificial conditions. In experiments with whole blood the possibility always exists that the differences observed may depend upon mechanical factors related to differences in the character of the clot formed in the blood of different animals. We have, therefore, not employed whole blood, but have used a serum-leucocyte mixture and have kept the mixture in constant motion.

Method.

A method was devised for growth inhibition tests with the pneumococcus, whereby a constant thorough mixing of leucocytes and organisms was obtained during incubation. Mixtures of serum and

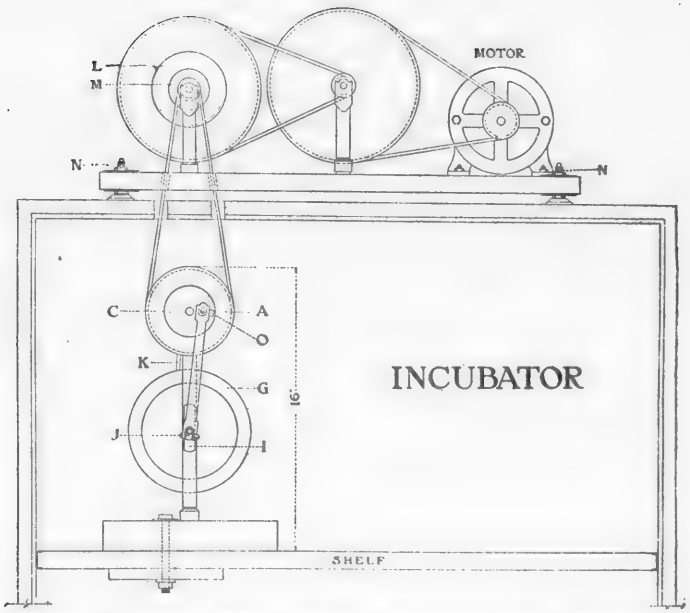
washed leucocytes in small glass tubes were seeded with varying numbers of pneumococci. The tubes were then sealed with paraffined corks, and attached to an agitating apparatus, placed in the incubator.[1]



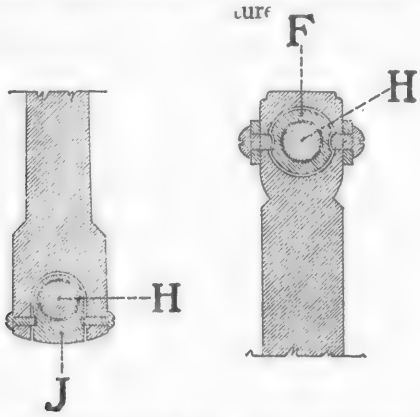
TEXT-FIG. 1. Diagram, drawn to scale, showing apparatus used for agitation. *A*, drive wheel (from motor); *B* and *E*, wheels driving belts from shaft *D* to shaft *H*; *C*, adjustable eccentric; *F*, central pivot-bearing; *G*, wooden wheels to which tubes are attached; *I*, slot in which shaft, *H*, oscillates.

The small drive wheels *B* and *E* are pictured the same size. This is incorrect. *B* wheels are 1 inch in diameter while *E* wheels are 1¼ inches in diameter.

[1] Rosenau (Rosenau, E. C., *J. Infect. Dis.*, 1906, iii, 683) describes a method whereby tubes or capillary pipettes containing serum, cells, and pneumococci were shaken in a machine in the incubator. He obtained more constant findings on bactericidal action than when no agitation was employed, but noted a reduction of the number of organisms, not the death of all. The tubes were not sealed; no description is given of the apparatus, and control tests were not provided.



TEXT-FIG. 2. Diagram, drawn to scale, showing apparatus mounted in incubator. *J*, pivot-bearing connecting eccentric with shaft *H; K*, drive belt from *BB* to *EE* (Fig. 1); *L* and *M*, pulley wheels for speed changes; *N*, screw adjustment for tightening belt from pulley system to drive wheel, *A*. The incubator shelf is an ordinary perforated wire shelf through which a bolt passes fixing the base of the apparatus. Purchase for the bolt is obtained with a block of wood.



TEXT-FIG. 3. Drawing showing in detail central pivot-bearing, *F*, and eccentric shaft bearing, *J* (see Text-figs. 1 and 2).

Apparatus.—The apparatus pictured in Text-figs. 1, 2, and 3 and Fig. 1 consists of two solid wooden wheels, equidistant from a central bearing, *F*, on an axle made to oscillate in a vertical plane by means of an adjustable eccentric. The apparatus is driven by a small motor, 1/12 h.p., the speed being reduced to the desired rate by means of a series of pulleys. The speed reduction may also be made by means of a worm-drive direct from the motor to the drive wheel (*A*) of the apparatus. The wheels and eccentric are mounted on a block and placed inside the incubator, with the motor and pulleys on top or above it. The small drive wheels, *EE*, have a diameter one-fifth larger than the wheels, *BB*. From this results a difference in the speed of oscillation and rotation. Two driving belts, *B*, *E*, are employed, because otherwise the small oscillation of the shaft at *E* produces sufficient slackness of the belt at the height of the upward movement to cause slipping. One or the other of the two belts, placed as in the figure, is always tight, thereby insuring constant motion. A flat leather belt, with a piece of tape run through a series of slits cut in the leather (Fig. 1) serves as the holder for the tubes. The means by which the loaded belt is held on the wheel is shown in the illustration. The tape passes under a small metal rod and is then wound on a shaft mounted half an inch above the surface of the wheel and perforated to hold the end of the tape. A rachet wheel with a small detachable handle keeps it in place. It is a simple matter to carry different numbers of tubes in the belt, since the slack of the tape can be taken up.

When the apparatus is in operation, the motion given to the tubes —rotation plus oscillation at a different rate of speed—serves to mix their contents thoroughly and keeps inner surface of the tubes moist, an important matter, because drying would permit some of the organisms to escape phagocytosis. Rotation and oscillation are carried on slowly, so as to reduce to a minimum mechanical injury to the leucocytes.

Constituents of the Test.

Preparation of Organisms.—The growth of the pneumococci, preparation of the standard solution, and method of making the dilutions have been described in the preceding publication (8). Gelatin-Locke's solution, prepared and adjusted to pH 8.0 with $NaHCO_3$, was the suspension fluid.

Strain of Pneumococcus Used.—The organism employed in all the following experiments except 10 and 11, was of Type I, originally isolated from a case of lobar pneumonia and kept in blood broth for several years. Just prior to the experiments the culture was passed through four guinea pigs in quick succession and its virulence for rabbits, guinea pigs, and cats tested, with the following results. Rabbits, 0.00001 cc. of the standard suspension killed; guinea pigs, 0.001 cc. of a heavy broth killed; cats, 1 to 3 cc. of the standard suspension were required to

kill animals of about 2,000 gm. The Type III pneumococcus of Experiments 10 and 11 was one recently isolated from the peritoneal exudate of a case of pneumococcus peritonitis. 4 cc. of the standard suspension killed a 2,000 gm. cat at the end of 10 days. Care was taken that the culture used in the experiments was always in the active growth phase.

Serum.—The serum was obtained for the most part from normal adult animals, by cardiac aspiration. The blood was placed in centrifuge tubes, allowed to clot at room temperature, separated from the walls of the tube, and centrifuged. The serum was removed immediately and kept overnight in the ice box. Occasionally serum was procured on the day of the test. During the latter part of the work, partly for the sake of economy, serum from the animal supplying the leucocytic exudate was used. It was thought that by this means possible untoward effects from blood incompatibility would be avoided. But no difference in results could be made out. Tests for isoagglutinins and isohemolysins were not performed as routine, but in certain instances where they were carried out no evidence of incompatibility was found.

Leucocytes.—The leucocytes were usually obtained from pleural exudate produced by aleuronat, though occasionally leucocytes separated from the circulating blood were employed, as noted in the protocols. 5 cc. of a mixture of equal parts of aleuronat suspension[2] and broth were injected into each pleural cavity of a cat or a rabbit, the species chiefly used in our work. After 24 hours the chest and abdomen were shaved under ether, 5 per cent carbolic acid applied and blood taken for serum and an erythrocyte suspension. For this latter the blood was mixed with an equal quantity of 1 per cent sodium citrate in 0.9 per cent NaCl solution. The animal was then killed with ether, both pleural cavities opened under sterile precautions and 5 to 6 cc. of salt-citrate solution allowed to run into the pleural cavity, the contents of which were transferred to a 15 cc. graduated centrifuge tube while the exudate left behind was taken up with more salt-citrate. In the distribution of it into centrifuge tubes care was taken that the salt-citrate solution was present in at least an equal volume and well mixed with it. A count of the leucocytes was now made on the tube containing the heaviest exudate. 0.1 cc. of the well distributed suspension was taken up into a 0.1 cc. pipette and added to 0.4 cc. salt-citrate solution in a test-tube 1 × 10 cm. containing a glass bead. The pipette was washed out by drawing up the fluid from the test-tube several times. 0.5 cc. of a 0.5 per cent acetic acid solution was next added and after shaking the tube for 5 minutes the leucocytes were counted in the usual way. The standard quantity used in this work was 0.5 cc. of a suspension containing 10,000 leucocytes per c.mm. To determine the number of serum-leucocyte tubes that the total leucocyte suspension would provide, a simple calculation sufficed.

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[2] The aleuronat suspension is prepared as follows: A solution of 1.5 gm. of starch in 75 cc. of water is brought to a boil and 5 gm. of finely powdered aleuronat are slowly added, with constant stirring.

$$\frac{\text{Leucocyte count of suspension}}{10{,}000} \times 2 \times \text{cc. of suspension} = \text{number of serum-leucocyte tubes.}$$

The leucocyte suspensions were next centrifuged at about 1,000 rotations per minute, for 7 to 8 minutes, following which the cells were washed twice, first in gelatin NaCl 0.9 per cent solution, then in gelatin-Locke's solution. Gelatin-containing solutions were used because of Rous and Turner's (9) finding that a small quantity of gelatin served to protect red blood cells against injury when washed. Although these authors did not find that the phagocytic activity of the leucocytes was diminished after washing in non-gelatin-containing solutions, it is possible that the much more highly specialized function of intracellular digestion may well be disturbed thereby. When the yield of leucocytes was small the contents of two or more tubes were combined during the washing. Following it the suspension was made up to a volume such that 0.1 cc. contained 5,000 leucocytes. This concentrated suspension, however, was not made up until after the addition of red blood cells, to be described shortly.

In order to procure leucocytes from the circulating blood, animals were bled from the heart and 4 to 5 cc. of blood distributed into each of several 15 cc. centrifuge tubes containing 6 to 8 cc. of salt-citrate solution, and well mixed. The tubes were then centrifuged for 10 minutes at low speed, the supernatant fluid removed, the leucocyte layer taken in a capillary pipette and transferred to one 15 cc. graduated centrifuge tube, suspended in gelatin-salt solution, and a count made. The later procedure was the same as with leucocytes from an exudate. The red cells were washed in the same manner. 1 cc. of rabbit's or cat's blood will yield enough leucocytes for only one tube or less. From each cc. of guinea pig's blood, on the other hand, sufficient leucocytes can usually be obtained for two tubes.

Red Blood Cells.—Red cells were used as an indicator of bacterial growth. Under conditions of constant agitation, the growth of pneumococci in the presence of blood regularly changes oxyhemoglobin to methemoglobin; and the lack of the change indicates an absence of growth. Given a suitable amount of red blood cells, 0.04 to 0.05 cc. to each tube, the extent of the color change paralleled very closely the degree of growth of the pneumococci during the first 24 to 30 hours of culture. Thereafter color changes from other causes may occur. To insure an identical amount of red cells in each test, the sediment of them, from which the leucocyte layer had been removed, was added directly to the washed leucocyte sediment, which latter usually contained some erythrocytes. The red cells were added in a sufficient quantity to make up a bulk equal to half the volume of the ultimate leucocyte suspension, which was then brought to its final volume with gelatin-Locke's solution.

When for any reason there was a delay in mixing, the leucocytes were placed on ice, whereas the suspension of organisms was kept at room temperature because cold causes the organisms to go into a state of lag. In the early part of the work no attention was paid to the hydrogen ion concentration of the solutions used for

CORRECTION.

In Vol. xxxix, No. 2, page 226, line 12, for *0.1 cc. contained 5,000 leucocytes* read *0.1 cc. contained the calculated number of leucocytes for one tube.*

washing and suspending the leucocytes. They were made up from freshly double distilled water sterilized in hard glass flasks and used soon afterwards. Recent work by Evans (10) has shown that the phagocytic activity of leucocytes is depressed by suspension in acid solutions. In the later experiments, therefore, gelatin-salt and gelatin-Locke's solutions were adjusted to a pH 7.2 to 7.5 by the addition of $NaHCO_3$, as described in the previous paper, though with this difference, that no phosphate was added to the gelatin-salt solution. The salt-citrate was found to have a reaction of about pH 7.8 and was used without further adjustment.

Assembling the Test.

Preparation of Small Tubes.—The test-tubes employed were of moderately thin walled glass, 6 cm. long by 7 mm. inside diameter. They were soaked overnight in cleaning fluid, rinsed thoroughly with tap water, boiled in twice distilled water for ½ hour, and scrubbed with hot water and soap. After a second rinsing they were boiled in twice distilled water again for ½ hour, and dried and plugged with cotton. Instead of dry sterilization, the tubes contained in large Petri dishes were autoclaved at 15 pounds pressure for 1 hour and the steam quickly released at the end of this time. Complete drying was accomplished by placing them in a drying oven at 60°C. for 1 hour. Dry sterilization was avoided because not infrequently a hemolytic oil from the cotton is deposited on the walls of the tube when the temperature rises over 140°C., while at times even lower temperatures may cause this to occur.

Preparation of Corks.—Snugly fitting corks with a smooth lower face were chosen for sealing the tubes. They were boiled in twice distilled water for 1 hour, dried, and sterilized in glass containers as with the small tubes, and like them placed in the oven to dry.

Mixing.—The test ingredients were mixed in the following quantity and order.

Serum,	0.2 cc. or 0.3 cc.	+	Leucocyte suspension (containing red blood cells), 0.1 cc.[3]	+	Suspension of organisms,	0.1 cc.

In handling the organisms the highest dilution was added first to all the tubes of that dilution. The same pipette was used throughout and the last 0.1 cc. in the pipette was not delivered. After distributing the organisms, the open end of the tube was flamed thoroughly while held nearly horizontal and was sealed with a cork dipped in hot paraffin. The tube was then rotated rapidly, which allowed the small amount of paraffin carried over on the cork to form a ring at the junction of cork and glass. If the ring failed to form, the cork was redipped. Blowing gently on the cork during rotation hastens the solidification of the paraffin.

[3] Where leucocytes or red blood cells were omitted in the control tubes an equivalent volume of gelatin-Locke's solution was added.

The tube should be flamed only briefly before insertion of the cork, since it is undesirable to have the paraffin run down the inside of the tube. As each tube was placed in the belt it was inverted in order to determine whether or not the sealing was effective. At the same time it was agitated gently so as to moisten the entire inner surface. If too much paraffin is carried in with the cork, the blood will not flow back and forth on tilting. In this event it is desirable to transfer the tube's contents to a fresh tube.

After the test was completely assembled, controls on the organism suspension were planted. 0.1 cc. of the sixth dilution (0.0000001 cc. of the standard suspension) was inoculated into each of two tubes containing 4 to 5 cc. of 1 per cent dextrose blood broth, pH 8.0. Horse blood or rabbit blood was used—0.1 cc. of blood in each tube. If the organisms were in the proper growth phase at the time the suspension was made, abundant growth occurred in 12 to 15 hours. Failure to grow indicated that the suspension was not suitable for the test, and the results were therefore discarded.

The rabbit blood agar plates employed were also tested for their suitability as culture medium. One or two controls were always made on the fluid portion of the medium, *i.e.* serum plus gelatin-Locke's solution, to show that these ingredients of the test mixtures did not cause growth inhibition. Repeated virulence tests of the culture used showed that as small quantities as 0.0000001 or 0.00000001 cc. of the standard suspension always killed mice.

In the majority of the early experiments agitation was maintained at about 18 to 20 revolutions and 8 to 10 oscillations per minute,[4] but in some of the later tests at 15 to 20 per minute. (See page 238 for optimum rate of agitation.) The eccentric was so adjusted that the shaft, *H*, oscillated in the slit, *I*, through a distance of approximately 1½ inches, or an arc of 10 to 11°. The incubator was maintained at 36–37°C. A small electric fan placed inside served to keep an even temperature throughout it. The tubes were observed several times during the first hour's agitation, since there is a tendency for small bubbles to collect at the cork end of some, owing to an excess of paraffin. Their presence is obviously undesirable. A gentle tap will dissipate them, but if they recur persistently, the contents of the tube should be transferred to a fresh tube.

Method of Determining Results.—In determining the results of the tests: (*a*) stained films were made from the tubes to determine whether bacteria were present microscopically; (*b*) cultures were made to determine whether any living bacteria were present; (*c*) in certain instances this was controlled by injection of a definite portion of the contents of the tubes into susceptible mice; (*d*) the change in color of the hemoglobin in the red blood cells in the mixture, due to the formation of methemoglobin, was also used as an indicator of growth of the pneumococci present.

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[4] The experiments in the first part of this work were performed with an agitator, other than that described, in which the ratio of rotation to oscillation rate could be varied at will. This was found to have no advantage over a fixed ratio.

EXPERIMENTAL.

Resistant Animals.

Experiment 1.—(Table I.) Cat serum and leucocytes from the circulating blood were used, procured on the day of the test. Continuous agitation was kept up for 72 hours; revolutions 18 to 20 and oscillations 8 to 10 per minute. The blood pigment changes noted during the first 24 hours. After 72 hours the tubes were opened and stained films carefully examined for pneumococci. The contents of each tube were then transferred to 4 to 5 cc. of 1 per cent dextrose blood broth pH 7.9 to 8.0. These cultures were examined at intervals of 6 to 12 hours during a period of 48 hours incubation.

TABLE I.

Cat serum 0.2 cc. + cat leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Color change. At hrs.			Pneumococci in stained film at 72 hrs.	Growth in culture at 72 hrs.
	12	18	24		
cc.					
0.01	++++			+	
0.001	0	±	++++	+	
0.0001	0	0	0	0	0
0.00001	0	0	0	0	0
0.000001	0	0	0	0	0
Control without leucocytes.*					
0.000001		++++		+	

Color changes indicating formation of methemoglobin are indicated as follows:

± = first appreciable change—a little darker red.
\+ = well marked change—definitely darker.
++ = moderately deep red.
+++ = very deep purplish red.
++++ = black.

* 0.05 cc. washed red blood cells freed from leucocytes and suspended in Locke's solution were added.

It is seen in Table I that color changes indicating bacterial growth occurred in Tubes 1 and 2 within 12 hours and 18 hours, respectively, but that with quantities of pneumococci representing less than 0.001 cc. of the standard suspension none took place. No organisms were found in these tubes microscopically at the end of 72 hours, and cultures of the tube's contents showed no growth.

In order to exclude the possibility that lack of growth in dextrose blood broth at the end of 72 hours might be due not to actual death of the pneumococci but to certain involutional changes brought about by the long inhibition of growth in the serum-leucocyte mixture, the tubes in Experiment 2 were cultured at the end of 24 hours.

TABLE II.

Cat serum 0.3 cc. + cat leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	At 24 hrs.			Culture media used.
	Color change.	Pneumococci in stained film.	Growth in culture.	
cc.				
0.01	++	+	+	Dextrose blood broth, 5 cc.
0.001	+	+	+	
0.0001	0	0	0	
0.00001	0	0	0	
0.000001	0	0	0	
0.01	+	+	+	Rabbit blood agar plates.
0.001	0	0	0	
0.0001	0	0	0	
0.00001	0	0	0	
0.000001	0	0	0	
0.01	++	+	+	Dextrose blood broth, 10 cc.
0.001	0	0	+	
0.0001	0	0	0	
0.00001	0	0	0	
0.000001	0	0	0	
Control with serum only.				
0.000001		+	+	

Experiment 2.—(Table II.) Cat serum and leucocytes used; revolutions 17 to 18 and oscillations 8 to 9 per minute; agitation continued for 24 hours. At the end of this time color changes were noted and the contents of the tubes examined microscopically and transplanted immediately into 1 per cent dextrose blood[5] broth pH 7.9 to 8.0 and also plated in rabbit blood agar pH 7.3, as indicated in the protocol. Plates were made with agar at a temperature of 40–41°C. A

[5] The 10 cc. tubes received 0.2 cc. horse blood each.

control plate made with 0.000001 cc. of the standard suspension showed good growth. The culture tubes and plates were observed at frequent intervals for 68 hours.

The results of this experiment (Table II) are identical with those of Experiment 1. The media used for culturing the tubes at the end of 24 hours was that used for testing the organism suspension. 0.0000001 cc. of the standard suspension seeded into 10 cc. dextrose blood broth gave an abundant growth in 16 hours, indicating that the medium was excellent for the growth of pneumococci. Part of the tubes was transferred into 10 cc. of the test medium to further dilute any inhibitory substance acting in the 5 cc. amounts. Cultures were also made with rabbit blood agar plates, since the possibility existed that a few surviving organisms might grow in this medium and fail to grow in broth. But as in the case of the tubes cultured in broth, all that showed no growth at the end of 24 hours were found to be sterile on plating.

In order to be certain that we were providing a sufficiently sensitive growth medium for testing the viability of the pneumococci, the contents of the tubes in the next experiment were injected into field mice, which had been found very susceptible to the organism used. 0.0000001 cc. injected into mice weighing 18 to 20 gm. killed regularly in 24 to 36 hours.

Experiment 3.—(Table III.) Cat serum and leucocytes used. Media controls were made immediately following the assembling of the test but there was a delay of some 2 hours before the tubes were started at constant agitation. Revolutions 11 to 12 and oscillations 9 to 10 per minute for 14 hours, then agitation slowed down to revolutions, 4 to 5 and oscillations, 4 per minute for the remainder of the 24 hours. At 24 hours color changes were noted, the contents of the tubes were examined microscopically and transplanted into 1 per cent dextrose blood broth, pH 8.0, rabbit blood agar, pH 7.8, and small field mice as indicated in the table. Those tubes showing marked color changes were not cultured. The mice were observed for 12 days.

The results of this experiment show that pneumococci the growth of which had been inhibited in the cat serum-leucocyte mixture cannot be recovered even by injection into such a sensitive culture medium as the mouse. In a repetition of part of this experiment, it was found that 10,000 times the number of pneumococci sufficient

to kill a mouse, failed to kill after a 24 hour sojourn in the cat serum-leucocyte mixture. The experiment also indicates that dextrose blood broth is at least as sensitive an indicator of the viability of the pneumococcus as is the mouse.

TABLE III.

Cat serum 0.3 cc. + cat leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	At 24 hrs.			Culture media used.
	Color change.	Pneumococci in stained film.	Growth in culture.	
cc.				
0.01	++++	+		Field mice, weight 20 gm.
0.001	0	+	D. 24–36°.	
0.0001	0	+	" 41°.	
0.00001	0	0	Survived.	
0.000001	0	0	"	
0.01	++++	+		Dextrose blood broth.
0.001	+	+	+	
0.0001	0	+	+	
0.00001	0	0	0	
0.000001	0	0	0	
0.01	++++	+		Rabbit blood agar plates.
0.001	+	+	+	
0.0001	0	0	+	
0.00001	0	0	0	
0.000001	0	0	0	
Controls.	Media.			
0.0000001	Serum.	+		
"	Dextrose blood broth.	+ (14°).		
"	Blood agar plate.	+ 80 colonies.		
0.000001	Mouse.	D. 24–36°.		
0.0000001	"	" 24–36°.		

The question arises as to whether or not the findings constitute conclusive proof that the pneumococci were destroyed by the serum-leucocyte mixture. The sole test for the viability of microorganisms in such small numbers lies in their ability to grow. The failure of

the pneumococci of these experiments to show growth in even the most favorable media may be interpreted as signifying death of the organisms.

TABLE IV.

Dog serum 0.3 cc. + dog leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	At 24 hrs.		
	Color change.	Pneumococci in stained film.	Growth in culture.
cc.			
0.01	++++	+	
0.001	++	+	
0.0001	0	0	0
0.00001	0	0	0
0.000001	0	0	0
Control with serum only.			
0.000001		+	

TABLE V.

Rabbit serum 0.3 cc. + rabbit leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	At 24 hrs.		
	Color change.	Pneumococci in stained film.	Growth in culture.
cc.			
0.001	+++	+	+
0.0001	++	+	+
0.00001	++	+	+
0.000001	+	+	+
0.0000001	+	+	+
Control with serum only.			
0.0000001		+	

In order to determine whether the blood of other animals resistant to pneumococcus infection possesses pneumococcidal properties, tests were carried out with the serum and leucocytes of the dog.

Experiment 4.—(Table IV.) Dog serum used and leucocytes obtained from the circulating blood; rates of oscillation, 8 to 9 and rotation, 17 to 18 per minute, but agitation continued for only 6 hours; tubes left undisturbed until the end of 24 hours, when they were examined microscopically and cultured in 1 per cent dextrose blood broth pH 7.9, in which 0.0000001 cc. standard suspension grew abundantly in 16 hours.

The pneumococci failed to grow in mixtures of dog serum and leucocytes in quantities smaller than 0.001 cc. of the standard suspension, or about 1 million organisms; that is to say, there was approximately the same degree of growth inhibition as in the case of the cat.

Susceptible Animals.

Experiment 5.—(Table V.) Rabbit serum and leucocytes used; revolutions, 17 to 18 and oscillations, 8 to 9 per minute; agitation continuous for 24 hours, at the end of which time the tubes were examined microscopically.

It is seen that rabbit serum and leucocytes fail to cause any growth inhibition of even such a small number of pneumococci as 0.0000001 cc. The same suspension of organisms was used in this experiment as in Experiment 2, and the tests were run simultaneously under identical conditions.

In order to determine whether a larger number of leucocytes in the rabbit serum-leucocyte mixture would be more effective in causing growth inhibition, double the quantity of them was used in the next experiment. Also a duplicate set of tubes was put up without red cells, so as to permit possibly a more intimate contact between leucocytes and organisms.

Experiment 6.—(Table VI.) Rabbit serum and leucocytes; suspension of leucocytes twice as concentrated as in previous experiments; revolutions, 18 to 20 and oscillations, 8 to 9 per minute; agitation continuous for 24 hours, at the end of which time color changes noted and stained films made from the tubes.

Increasing the number of leucocytes in the rabbit serum-leucocyte mixture failed to produce any growth inhibition of pneumococci.

Experiment 7.—(Table VII.) Guinea pig serum and leucocytes from the circulating blood were compared with rabbit serum and leucocytes. The same suspension of organisms was used in both, and the tests were carried out simultaneously

under identical conditions. Oscillations, 8 to 10 and revolutions, 30 to 40 per minute; agitation continuous for 48 hours; color changes noted at frequent intervals for 48 hours; and at the end of 70 hours incubation the tubes were opened and the contents examined microscopically.

While growth of the pneumococcus in the guinea pig serum-leucocyte mixture was found to be much slower than in the rabbit elements, all the tubes ultimately showed growth. The growth retarding effect may lie in the guinea pig serum alone, since the rabbit serum control grew out much earlier than did the guinea pig serum control.

TABLE VI.

Rabbit serum 0.3 cc. + rabbit leucocyte suspension (double concentration with and without red blood cells) 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Red blood cells.	At 24 hrs.	
		Color change.	Pneumococci in stained film.
cc.	*cc.*		
0.001	0.05	+++	+
0.0001	"	+++	+
0.00001	"	+++	+
0.000001	"	+++	+
0.0000001	"	0	+
5 dilutions as above.	0	*	+
Control with serum only. 0.0000001			+

* No color change on account of lack of red blood cells.

In order to exclude the possibility that failure of the rabbit and guinea pig serum-leucocyte mixtures to cause growth inhibition of the pneumococcus might be due to injury to the leucocytes resulting from prolonged agitation, an experiment was carried out in which the tubes were agitated for only 6 hours.

Experiment 8.—(Table VIII.) Growth inhibiting effect of serum and leucocytes of rabbit, guinea pig, and dog compared. The leucocytes were kept on ice for 24 hours before use. They were suspended in their homologous serum, except the dog leucocytes, which were by mistake suspended in Locke's solution. Oscillations, 8 to 10 and revolutions, 16 to 20 per minute; agitation for 6 hours;

TABLE VII.

Normal serum 0.3 cc. + leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Media.	Amount of standard suspension.	Color change. At hrs. 14	16	18	20	24	27	42	48	Pneumococci in stained film at 70 hrs.
	cc.									
Guinea pig serum and leucocytes.	0.01	++	++++							+
	0.001		+	++	+++	++++				+
	0.0001			±	±	++	++++			+
	0.00001						±	+++	++++	+
	0.000001							+++	++++	+
	0.0000001							++	++++	+
Rabbit serum and leucocytes.	0.001	++++								+
	0.0001	++	++++							+
	0.00001		++	+++	++++					+
	0.000001			+	+++	++++				+
	0.0000001			±	+++	++++				+
	Controls without leucocytes.*									
Guinea pig serum.	0.0000001						+	++++		+
Rabbit serum.	"			+	++++					+

* 0.05 cc. washed red blood cells suspended in equal quantity of Locke's solution added to control tubes.

then tubes left undisturbed for 24 hours, at the end of which time films were made and cultures of those tubes showing no growth were planted in 1 per cent dextrose blood broth pH 7.9 to 8.0.

TABLE VIII.

Normal serum 0.3 cc. + leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Media.	Amount of standard suspension.	At 24 hrs.		
		Color change.	Pneumococci in stained film.	Growth in culture.
	cc.			
Rabbit serum and leucocytes.	0.001	+++	+	
	0.0001	++	+	
	0.00001	++	+	
	0.000001	+	+	
	0.0000001	+	+	
Guinea pig serum and leucocytes.	0.01	++	+	
	0.001	+	+	
	0.0001	0	+	
	0.00001	0	+	
	0.000001	0	0	+
	0.0000001	0	+	+
Dog serum and leucocytes.	0.01	++++	+	
	0.001	++	+	
	0.0001	0	0	0
	0.00001	0	0	0
	0.000001	0	0	0
	Controls without leucocytes.			
Rabbit serum.	0.0000001		+	
Guinea pig serum.	"		+	
Dog serum.	"		+	

The results of Experiment 8 show that with 6 hours agitation pneumococci failed to grow in dog serum-leucocytes in quantities less than 0.001 cc., whereas in rabbit and guinea pig serum even 0.0000001 cc. of the standard suspension showed growth at the end of 24 hours. The first part of the experiment was repeated, using freshly obtained rabbit leucocytes. The result was the same.

Further Observations on the Action of Cat Serum and Leucocytes.

The observations were extended to determine more about the individual factors in the results. For the purpose cat serum and leucocytes were employed in the quantities used previously.

1. Rate of Agitation Necessary.—The speeds of oscillation and rotation were varied over fairly wide limits, and those finally adopted as most suitable were oscillations, 15 to 20 and revolutions, 18 to 24 per minute. However, a little slower speed than this still appears to bring about effective contact between pneumococcus and leucocyte while somewhat more rapid agitation does not seem to injure the leucocytes if the agitation is not continued too long. With very slow agitation on the other hand—4 to 6 revolutions and oscillations per minute—the degree of growth inhibition was definitely decreased.

2. Length of Time of Agitation Required. Experiment 9.—(Table IX.) Tubes agitated at rate of revolutions, 24 and oscillations, 20 per minute. After agitation each set of tubes except the last was taken and allowed to stand in the incubator for 24 hours when they were opened, the contents examined microscopically and transplantations made into dextrose blood broth from those showing no growth. The last set of tubes was agitated continuously for 72 hours. The H ion concentration of the broth in all these experiments was pH 7.8 to 8.0.

Table IX shows that in a relatively short time the maximum degree of phagocytosis is effected, as shown by the fact that growth inhibition in tubes agitated for 2 hours was as great as in those agitated for 24 hours. It might be concluded from the result in the 72 hour tubes that continuous agitation for this length of time was advantageous; but it has been found that a certain amount of variation sometimes occurs within the same experiment as shown in Table X, in which tubes agitated for 18 hours showed more inhibition of growth than those agitated for a longer period of time.

3. Rate of Bactericidal Action. Experiment 10.—(Table X.) Type III pneumococcus used; revolutions, 24 and oscillations, 20 per minute; contents of tubes cultured at intervals of time from 3 to 72 hours; agitation continued for 9 hours. Those tubes cultured at periods later than 9 hours were left undisturbed in the incubator until the time of culture. The tubes cultured at intervals earlier than 24 hours were not examined microscopically.

It is realized that the results of Experiment 10 do not constitute conclusive proof that bactericidal action begins to occur as early as 3 hours after placing the pneumococci in contact with the leucocytes.

It would be possible to prove this point only by injuring the leucocytes sufficiently to liberate the ingested organisms. However, the fact that no special precautions were taken in transferring the serum-leucocyte-pneumococci mixtures into the dextrose blood broth, combined with the finding that bactericidal action became progressively more pronounced as the time went on, reaching a maximum somewhere between 9 and 18 hours, would indicate that lack of growth in the culture signified that even at 3 hours, death of the organism was taking place. A repetition of the experiment gave similar results.

TABLE IX.

Effect of Period of Agitation.

Cat serum 0.3 cc. + cat leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Length of time agitated.	At 24 hrs.		
		Color change.	Pneumococci in stained film.	Growth in culture.
cc.	*hrs.*			
0.001	2, 3, 6, 9, respectively.	+	+	
0.0001		0	0	0
0.00001		0	0	0
0.000001		0	0	0
0.0000001		0	0	0
0.001	24	0	0	+
0.0001		0	0	0
0.00001		0	0	0
0.000001		0	0	0
0.0000001		0	0	0
			At 72 hrs.	
0.001	72	0*	0	0
0.0001		0	0	0
0.00001		0	0	0
0.000001		0	0	0
0.0000001		0	0	0
Controls with serum only.				
0.000001			+	
0.0000001			+	

* Slight hemolysis in all these five tubes.

TABLE X.

Rate of Bactericidal Action.

Cat serum 0.3 cc. + cat leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Cultured at	Pneumococci in stained film.	Growth in culture.
cc.	*hrs.*		
0.001	3	−	+
0.0001		−	+
0.00001		−	+
0.000001		−	0
0.0000001		−	0
0.001	6	−	+
0.0001		−	+
0.00001		−	0
0.000001		−	0
0.0000001		−	0
0.001	9	−	+
0.0001		−	+
0.00001		−	0
0.000001		−	0
0.0000001	(Agitation stopped.)	−	0
0.01	18	−	+
0.001		−	0
0.0001		−	0
0.00001		−	0
0.000001		−	0
0.01	24, 48, 72, respectively.	+	
0.001		+	
0.0001		0	0
0.00001		0	0
Controls without leucocytes.			
0.000001		+	
0.0000001		+	

4. Quantity of Serum Required. Experiment 11.—(Table XI.) Type III pneumococcus employed. Revolutions, 24 and oscillations, 20 per minute; agitation continued for 9 hours only and tubes left undisturbed until 36 hours when they were agitated for 1 hour in order to bring out color changes. Those

that showed a change in color were then examined microscopically, and at 72 hours stained films were made of all the tubes. The contents of the tubes were not cultured.

It was found that the quantity of serum used in the previous experiments was considerably in excess of that actually needed. 0.05 cc. serum diluted to 0.3 cc. with gelatin-Locke's was just as

TABLE XI.

Varying the Quantity of Serum.

Cat serum 0.3 cc. + cat leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Quantity of serum.	Quantity of gelatin-Locke's.	Pneumococci in stained film at 72 hrs.
cc.	*cc.*	*cc.*	
0.01	0.3	0	+
0.001	0.3	0	+
0.0001	0.3	0	0
0.00001	0.3	0	0
0.001	0.2	0.1	+
0.0001	0.2	0.1	0
0.00001	0.2	0.1	0
0.000001	0.2	0.1	0
0.001	0.1	0.2	+
0.0001*	0.1	0.2	+
0.00001	0.1	0.2	0
0.000001	0.1	0.2	0
0.001	0.05	0.25	+
0.0001	0.05	0.25	0
0.00001	0.05	0.25	0
0.000001	0.05	0.25	0
Controls without leucocytes.	Serum.		
0.000001	0.1	0.2	+
"	0.05	0.25	+

* 0.15 cc. suspension added through error.

effective when employed in the serum-leucocyte mixture, as whole serum. Higher dilutions of serum were not made in Experiment 11 as it was feared that a medium containing under 20 per cent serum might prove a less suitable one for the growth of pneumococci.[6]

[6] The action of leucocytes suspended in inactivated serum will be dealt with in a subsequent communication.

5. Quantity of Leucocytes Required.—The amount of leucocyte suspension employed in these tests was chosen arbitrarily before any experiments on the influence of the leucocyte quantity had been performed. Later it was found that the number of leucocytes employed was often considerably in excess of the necessary one. However, the effectiveness of the leucocytes appeared to vary with the individual animal providing them. In one experiment, a tenth of the customary quantity of leucocytes sufficed to bring about maximum inhibition of growth. In other experiments this small quantity was found insufficient, and at times the employment of a leucocyte suspension containing even one-half the usual number of leucocytes, 5,000 per c.mm., failed to cause as much growth inhibition as occurred in other cases with a suspension containing 10,000 leucocytes per c.mm. Using twice the standard quantity failed to produce any increased inhibition of growth. The question of variations in the potency of leucocytes from different individuals of the same species is being investigated further.

6. Amount of Agitation and Time of Incubation Finally Adopted.—By means of a large number of tests the fact was determined that agitation for 6 hours at a speed of 15 to 20 oscillations, through an arc of 10 to 11°, and 18 to 25 revolutions per minute, brought about adequate contact between organisms and leucocytes.[7] The tubes were allowed to remain undisturbed until the end of 36 hours, when they were agitated for 1 hour in order that color changes might be brought out in those in which growth had occurred. Such tubes were examined because sometimes pneumococci growing very early in the test will have autolyzed by 72 hours. The tubes were agitated again for several hours just before the end of the 72 hour period when color changes were noted and the tubes examined microscopically. Latterly, cultures of the tube's contents have not been made, since cultures of more than 100 tubes showing no growth microscopically at 72 hours were found to be uniformly negative.

It was not until this manuscript was practically completed that the work of Fenn (11) on the phagocytosis of solid particles, came to our attention. The method of agitation employed in this work is

[7] Very occasionally prolonged agitation seemed to result in a certain amount of leucocyte injury as evidenced by a lessened degree of growth inhibition.

similar in many respects to that used by Fenn. He rotated small tubes containing serum, leucocytes, and particles of carbon or quartz, on a drum in the incubator and found that phagocytosis was much increased by agitation. Tilting the tubes so that a bubble passed up and down the tube during rotation caused a further increase in the number of particles phagocyted, and he observed that within certain limits the degree of phagocytosis depended on the rate of agitation. Our work shows a general agreement with this finding, in that a certain speed of agitation was necessary in order to obtain a maximum inhibition of growth.

SUMMARY AND CONCLUSIONS.

Somewhat discordant results which have been reported by others who have investigated the property of the whole blood of resistant animals to cause inhibition of growth or death of pneumococci have led us to investigate this matter and to develop a new technique in which the conditions as they are present in the animal body are more nearly imitated. The observations already made have rendered it probable that phagocytosis plays some rôle in any destructive power for pneumococcus which whole blood possesses. We have, therefore, employed mixtures of serum and leucocytes in our tests, since when blood is coagulated the conditions become highly artificial. Furthermore, in order to imitate more nearly the conditions in the circulating blood the mixtures have been constantly, though gently, agitated. For this purpose a specially devised apparatus has been employed. The mixtures of serum and leucocytes have been inoculated with varying numbers of pneumococci in the active growth phase and after varying intervals of time the tubes containing the mixtures of serum, leucocytes, and bacteria have been opened, examined microscopically, and cultures made.

Employing this technique it has been found that the growth of pneumococci having low virulence for cats is markedly inhibited in mixtures of cat serum and cat leucocytes. It was impossible to recover pneumococci from the tubes showing no apparent growth, either when the contents were transplanted into various kinds of culture media, or when the contents were injected into mice of a variety highly susceptible to pneumococcus infection. 10,000 times

the number of pneumococci sufficient ordinarily to kill a mouse failed to do so after a 24 hour sojourn in the cat serum-leucocyte mixture. Mixtures of dog serum and leucocytes exert a similar action. The serum and leucocytes of animals susceptible to pneumococcus infection (rabbits and guinea pigs,) on the other hand, failed to injure pneumococci even in extremely small quantities.

These results indicate that the blood of resistant animals, at least of the dog and cat, possesses destructive properties for pneumococci, and that this destructive power is not possessed by the blood of certain susceptible animals. The experiments suggest that natural immunity depends chiefly, if not entirely, upon this property. The leucocytes play an active part in this process, but whether the destruction of the pneumococci occurs entirely within the leucocytes or not is not determined. That the serum also plays a part is shown by the fact that when the serum of resistant animals was inactivated before being used in the serum-leucocyte mixture, the growth of even very small numbers of pneumococci was not prevented.

Further experiments with cat serum and leucocytes were carried out to determine the optimum rate and time of agitation, the amount of serum and leucocytes required, and also the period of incubation necessary for the inhibition of growth and death of the pneumococci to occur.

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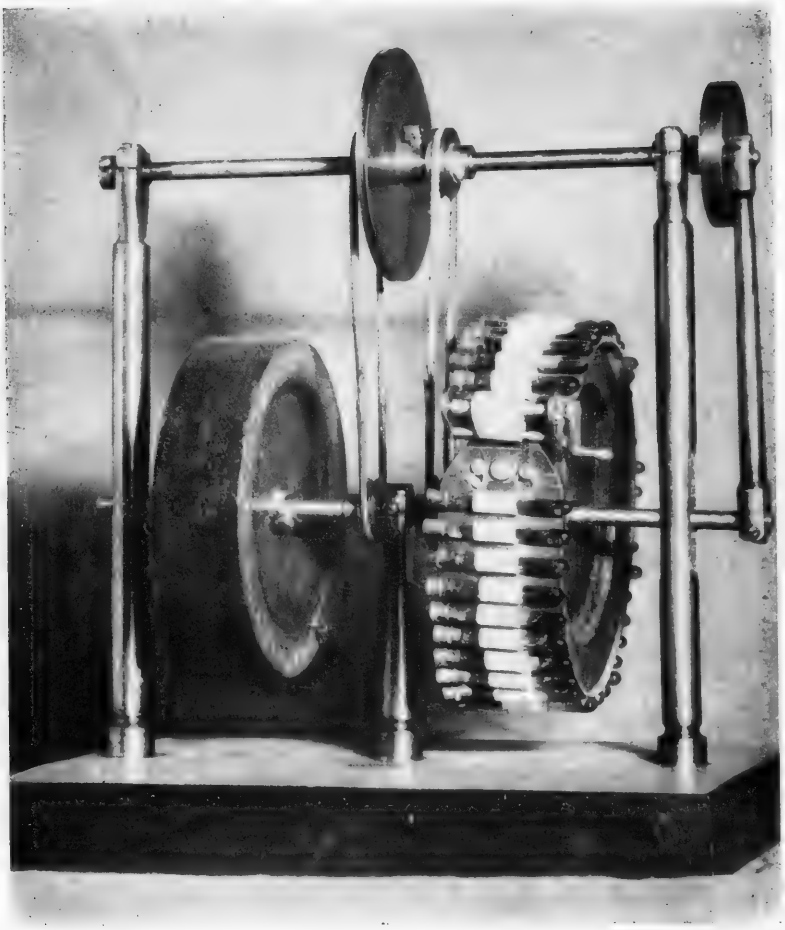
EXPLANATION OF PLATE 20.

FIG. 1. Agitator with belt carrying tubes attached.



(Robertson and Sia: Pneumococcus growth inhibition. II.)

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STUDIES IN AGGLUTINATION.

I. The Agglutination of Streptococci.

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INTRODUCTION.

In the immunological reaction of agglutination the character of the environment plays an important part. Among the influential factors are the conditions under which the bacteria are grown, the quality of the bacterial suspensions used, the temperature of incubation of the reaction, the nature and concentration of electrolytes utilized, and agitation or centrifugalization of the serum and bacteria mixtures. It is well known that departures from certain environmental requirements seriously interfere with the validity of the method. Optimal results may be obtained only if the environment be so standardized as to secure the most favorable state for the agglutination of any given organism.

The purpose of the present study is the determination of the more important factors essential for the production of an optimum agglutination balance. The importance and practical value of agglutination in diagnostic and immunological problems give particular impetus to such an investigation.

The mechanism of agglutination is usually conceived of as consisting of two phases: (*a*) union or interaction of antigen and antibody and (*b*) flocculation of the antibody-antigen complex. The first step may be accelerated by appropriate heat incubation (1), by agitation or centrifugalization (1), or by certain critical concentrations of electrolytes (2).[1] Interference with the interaction occurs when

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[1] As for example, the narrow pH zone just above the acid agglutination zone of any given organism, where the agglutinating power of the serum is increased (2).

the heat of incubation is excessive, when the concentration of hydrogen (3) or hydroxyl (4) ion is too high, when polyvalent ions are used as electrolytes,[2] or when such physical obstructive factors as bacterial capsules are present. The second phase is the basis for interpretation; interference with this step will therefore invalidate the method. The bacterial suspension may be too stable or too unstable. In the former case, evidence of antigen-antibody interaction will be lacking (except when cross-adsorption tests are possible), and in the latter case the occurrence of spontaneous or non-specific flocculation makes readings difficult or impossible. Here important factors are the character and concentration of electrolytes employed, and probably, the conditions of bacterial growth.

The optimum agglutination balance is secured, therefore, (1) when conditions are most favorable for antigen-antibody interaction, and (2) when the bacterial suspension is stable enough to prevent non-specific effects, and yet near enough to the flocculation threshold to be brought down by minimum concentrations of specific antisera.

As an approach to the problem, many forms of specific and non-specific agglutination have been studied. Here have been included the effect of hydrogen and hydroxyl ions, of monovalent and polyvalent ions (2, 3, 5–8) and the behavior of autoagglutinating bacteria. In addition, a survey of the methods commonly employed in the agglutination reaction has been made. Colon bacilli and streptococci have been employed chiefly in the experiments, but many other organisms have been used.

The Agglutination of Streptococci.

Most workers with streptococci who have utilized the methods of agglutination and adsorption in their problems have felt that their results might, perhaps, be considered equivocal at times, because of the difficulty of getting satisfactory suspensions of all strains of the organism. The strains vary from extremely granular types, with which it is impossible to work, to entirely satisfactory diffuse ones; and frequently, diffuse strains become granular, and granular ones become diffuse. When one is dealing with granular autoagglutinating strains, it is often impossible to make satisfactory readings of specific agglutination, or to interpret accurately related adsorption reactions. In drawing general conclusions regarding streptococcal types, as in the work on influenzal, empyema, or scarlatinal strains (with which agglutination and adsorption tests are used as the basis of interpretation), it becomes necessary, therefore, that methods be devised for obtaining uniformly stable suspensions of the bacteria.

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[2] Polyvalent ions precipitate the serum (*cf.* $CeCl_3$, personal observation).

In the present paper, a study has been made of the nature of streptococcal autoagglutination. On the basis of the experimental results, general conclusions regarding this phenomenon have been reached, and suggestions for the management of specific reactions have been put forward. The explanation ventured seems to be applicable, perhaps, to similar behavior of other bacteria.

Methods.

Strains of Streptococci.—Hemolytic strains, 10 from throats of scarlet fever, 5 empyema, 1 wound; non-hemolytic strains, 4 septicemia, 1 urine.

Serum.—Rabbit antistreptococcic serum.

Agglutination Methods.—The macroscopic method was used entirely.

Washing of bacteria: Bacteria were washed with distilled water two to four times.

Suspensions: The washed bacteria were suspended in distilled water, and a uniform concentration was sought by the Gates method (9) (opacity to equal a colon bacillus standard of 2 billion per cc.). At times the bacterial growth was small and lighter suspensions were perforce used.

Method of mixing: Equal quantities (usually 0.5 cc.) of the bacterial suspensions to be tested for agglutination, and of the solutions used, were mixed rapidly and thoroughly agitated.

Temperature of reactions: All agglutination tests were incubated in a water bath at 37°C., unless otherwise indicated.

Media.—Stock buffered beef broth, adjusted to a pH of 7.6, or to other pH as indicated.

Temperature of Growth.—Bacteria were grown at 37°C., and at room temperature (17–23°C.). Less bacteria were obtained at the latter temperature, but enough were grown when 40 to 100 cc. of broth were used.

Age of Cultures.—Bacteria were grown 16 to 18 hours; occasionally 24 hour growths were used.

Buffers.—The glycocoll, sodium phosphate, sodium acetate (G.P.A.) buffer recommended by Northrop and De Kruif (6), was found to be satisfactory and was used throughout.

Measurements of Potential.—Potential was determined from the rate of migration, by means of the Northrop cell (5). Calculations were made in accordance with Northrop's formula.[3]

Measurements of Cohesive Force.—Determinations were made by the method of Northrop and De Kruif (6). The du Noüy surface tension apparatus (10) was used.[4]

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[3] Use of the cell was kindly permitted us by Dr. Northrop of The Rockefeller Institute for Medical Research.

[4] Use of the apparatus was kindly permitted us by Dr. Northrop.

RESULTS.

In studying the acid agglutination zone of streptococci, it was noted that the zone varies from day to day, that it varies when the bacteria are grown in broth media of different pH, or in different batches of media, and that the zone varies at times for no accountable reason. It was also noted that there are marked differences in the width of the acid zones of different strains.

Stevens,[5] working in this laboratory, noted last year that granular strains of streptococci (37°C. growth) frequently became diffuse or less granular when incubated at room temperature. A similar related observation has been made for granular diphtheroids by Mellon (11) who believed that he was dealing with pleomorphism, because of the fact that he had two strains of his diphtheroid, one, smooth, predominating at room temperature (20°C.) and the other, granular, at 37°C. Judging from the results of agglutination tests, this does not seem true of the bacteria under our observation.

On the basis of these observations it seemed fair to assume that: (1) cultural conditions, notably thermal or H ion, may be so controlled as to reduce or prevent the tendency to granular growth, and (2) there may be a pH zone in which even very granular strains would remain sufficiently stable for carrying out serum reactions.

Tables I and II show the effect on macroscopic cultural characteristics, on the microscopic appearance of the bacteria, and on the acid agglutination zone of Strain ScB, when the bacteria are grown in broth of varying pH at room temperature and 37°C. respectively. There appear, also, in the tables, the changes in the pH of the broth that occur during growth.

It will be noted from Table I that (1) the growth at 37°C. is granular at lower pH the chains are long and the clumps of bacteria are large and frequent, and the pH of the broth drops a little more than in those grown at room temperature; and (2) growth at room temperature is diffuse in all cases, and the chains are short and the clumps small and infrequent.

From Table II it will be seen that the acid agglutination zone of Group 2 (grown at 37°C.) is regularly wider than that of Group 1, pH 3 to 9 as opposed to pH 3 to 5–6. Study of the rate of aggluti-

[5] Stevens, F. A., personal communication.

nation shows that this is more rapid in Group 2 than in Group 1, and that in Group 2 the rate is faster for the bacteria grown at a lower pH. It will be noted also that agglutination is not complete in Group 2 at pH 6.6 to 9.0. The bacteria grown at room temperature are stable in salt solution, NaCl M/14 (0.425 per cent), while all bacteria in Group 2 agglutinate.

Determinations of the pH at the end of the experiment show no differences sufficient to account for the results (Table III).

TABLE I.

Effect of Growth at Room Temperature and at 37°C., and of Varying pH on the Character of Streptococcus Cultures (Strain ScB).

Condition of growth.	Original pH of broth.	Final pH.	Gross growth characteristics.	Microscopic characteristics.
Bacteria grown 16 hrs. at room temperature.	7.20	6.80	Diffuse.	Chains short; clumps small and rare.
	7.35	7.00	"	" " " " " "
	7.55	7.10	"	" " " " " "
	7.60	7.35	"	" " " " " "
	7.70	7.40	"	" " " " " "
Bacteria grown 16 hrs. at 37°C.	7.20	6.70	Moderately granular.	" long; very many large clumps.
	7.35	6.80	Moderately granular.	" " " " " "
	7.55	7.00	Diffuse.	" " many large clumps.
	7.60	7.00	"	" " " medium sized clumps.
	7.70	7.10	"	" " " " " "

As noted above, the growth at room temperature is usually smaller in amount than that obtained at 37°C. To rule out the factor of earlier stage of growth, in the behavior of the two types, bacteria were grown at 37°C. until their density was the same as that of a room temperature growth begun earlier (density determination by Gates' (9) opacity method). These bacteria remain unstable. Also, in order to get more bacteria, growth was conducted at varying combinations of the two temperatures, starting at room temperature and ending at 37°C., and *vice versa*. The results here are again the same; *i.e.*, once the bacteria have been incubated at 37°C., they become unstable.

TABLE II.

Effect of Growth at Room Temperature and at 37°C., and of Broth of Varying pH on Agglutination of Streptococci at Various pH (G.P.A. Buffer).

Condition of growth.	pH of buffer.	Agglutination.														
		1 hr.					2 hrs.					24 hrs.				
		pH of broth.														
		7.2	7.35	7.55	7.6	7.7	7.2	7.35	7.55	7.6	7.7	7.2	7.35	7.55	7.6	7.7
Group 1; grown at room temperature.	3.0	1−	1	1−	1−	±	1	1+	1	1−	1+	C.	C.	C.−	C.	C.
	4.0	1−	1−	1−	1−	±	1	1	1	1−	1+	C.	C.	C.	C.	C.
	5.0	±	−	±	±	±	±	±	±	±	±	C.	C.−	C.	C.−	3
	6.0	−	−	−	−	−	−	−	−	−	−	C.−	±	C.−	±	±
	6.6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	7.0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	8.0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	9.0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Distilled H_2O	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	NaCl M/14	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Group 2; grown at 37°C.	3.0	3	3	1	1+	1	C.−	C.−	2	3	2+	C.	C.	C.	C.	C.−
	4.0	3+	3	2	3	2	C.−	C.−	3+	C.−	3+	C.	C.	C.	C.	C.
	5.0	3+	3	2−	3−	2−	C.−	C.−	3+	C.	C.−	C.	C.	C.	C.−	C.−
	6.0	3+	3	±	±	±	C.−	C.−	2	1+	1	C.	C.−	C.−	C.−	C.−
	6.6	3+	3	±	−	−	C.−	3+	2	±	−	C.	3+	3	2+	2
	7.0	3+	2+	±	−	−	C.−	3	2	±	−	C.	3+	3	2+	2
	8.0	3	2+	±	−	−	C.−	3	1+	±	−	C.	C.	C.	3+	3+
	9.0	2+	2	±	−	−	3	2+	1+	±	−	C.	C.	C.	3	3+
	Distilled H_2O	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	NaCl M/14	3	2+	1+	1+	−	C.−	3+	3+	2	1−	C.	C.	C.	C.−	3+

− indicates no agglutination; ±, doubtful agglutination, slightly granular with hand lens; 1, slight definite granular appearance of suspension to the naked eye; 2, marked granular appearance, without flocculation; 3, marked granular appearance, beginning flocculation; C., complete agglutination, supernatant fluid clear. + and − are used to indicate intermediates between these.

Similar acid agglutination zone determinations were now made with several strains, twenty in all. In all cases differences between bacteria grown at room temperature and at 37°C., similar to those noted above, are present. Examples of these differences are presented in Tables IV to VI.

Table IV shows the differences in the acid agglutination zones of strains in which there is a moderate tendency to granular growth, when the bacteria are incubated at 37°C. Table V shows the differences in a very granular strain; it will be seen here that the chief difference lies in the greater rapidity of agglutination of the bacteria grown at 37°C. Table VI shows the results when strains that are regularly diffuse are employed.

TABLE III.

Final pH of Mixtures at the End of the Preceding Experiment.

Readings made at the end of 24 hours.

pH of broth.	Temperature of growth.	Final pH.									
		pH of buffer used in mixtures.								Controls.	
		3.0	4.0	5.0	6.0	6.6	7.0	8.0	9.0	Distilled H_2O	NaCl
7.2	Room temperature.	3.0	4.0	5.0	6.0	6.7	7.0	7.8	8.4	6.6	6.5
	37°C.	3.0	4.0	5.0	5.9	6.6	6.9	7.9	8.4	6.5	6.5
7.35	Room temperature.	3.0	4.0	5.0	5.9	6.6	6.9	8.0	8.6	6.6	6.5
	37°C.	3.1	4.0	5.1	6.0	6.6	7.0	7.9	8.6	6.4	6.4
7.55	Room temperature.	3.1	4.2	5.0	6.0	6.6	7.0	7.8	8.4	6.4	6.4
	37°C.	3.0	4.2	5.0	6.0	6.6	7.0	7.8	8.4	6.4	6.4
7.6	Room temperature.	3.0	4.0	5.0	6.0	6.6	7.1	7.9	8.5	6.4	6.5
	37°C.	3.1	4.0	5.2	5.9	6.6	7.0	7.8	8.4	6.3	6.3
7.7	Room temperature.	3.0	4.0	5.0	6.0	6.6	7.0	7.8	8.4	6.5	6.5
	37°C.	3.0	4.0	5.1	6.0	6.6	7.1	7.9	8.5	6.5	6.4

Tables VII and VIII show the results when *Staphylococcus aureus* and *Bacillus typhosus* are treated in the same way. It will be noted that the stability of the staphylococcus is so increased by growth at room temperature that an acid agglutination zone is practically absent. Only slight differences appear in the acid zones of *Bacillus typhosus*.

TABLE IV.

Determination of the Acid Agglutination Zones of Moderately Granular Strains Grown at Room Temperature and at 37°C.

Readings made at the end of 24 hours.

Temperature of growth.	Strain.	Agglutination.									
		pH of mixtures.								Controls.	
		3.0	4.0	5.0	6.0	6.6	7.0	8.0	9.0	Distilled H_2O	NaCl
Room temperature.	N Y V	C.	C.−	3+	−	−	−	−	−	−	−
	D	C.−	C.	C.	1+	−	−	−	3*	−	−
	1372	C.	C.	C.	1+	−	−	−	−	−	−
	BL	C.	C.	±	−	−	−	−	†	−	−
37°C.	N Y V	C.	C.	C.−	3+	3+	3+	C.	C.	−	C.
	D	C.	C.	C.	C.	C.	C.	C.	C.	−	C.
	1372	C.	C.	C.	C.	C.	C.	2	C.−	−	C.
	BL	C.	C.	C.	C.	C.	C.	C.	†	−	C.

* Agglutination, usually not complete, has been noted many times in this alkaline zone (about pH 8.0 to 10.0). Experiments with G.P.A. buffer have shown that this zone of "alkaline agglutination" is present with many bacteria, *B. typhosus*, colon bacillus, staphylococcus, streptococcus, etc.

† Not done at this pH.

TABLE V.

Determination of the Acid Agglutination Zone of a Very Granular Strain (Strain H) Grown at Room Temperature and at 37°C.

pH of mixture.	Agglutination.							
	Bacteria grown at room temperature.				Bacteria grown at 37°C.			
	1 hr.	2 hrs.	6 hrs.	24 hrs.	1 hr.	2 hrs.	6 hrs.	24 hrs.
3.0	1	3	3+	C.	3	3+	C.−	C.
4.0	1	3	3+	C.	3	3+	C.−	C.
5.0	1	3+	C.−	C.	3	3+	C.−	C.
6.0	1−	2+	2+	C.	3	3+	C.−	C.
7.0	1−	3	C.−	C.	1	3+	3+	C.
8.0	−	2+	C.−	C.	−	2+	3	C.
9.0	−	−	3	C.	−	−	−	C.−
Distilled H_2O	−	−	−	−	−	−	−	−
NaCl	1+	3	C.−	C.	2+	C.−	C.	C.

TABLE VI.

Determination of the Acid Agglutination Zone of a Diffuse Strain (Strain SFI) Grown at Room Temperature and at 37°C.

pH of mixture.	Agglutination.							
	Bacteria grown at room temperature.				Bacteria grown at 37°C.			
	1 hr.	2 hrs.	3 hrs.	24 hrs.	1 hr.	2 hrs.	3 hrs.	24 hrs.
2.8	±	1−	1	C.−	1	2	2+	C.
3.6	±	1−	1	C.−	1	2−	2	C.
4.0	−	±	1−	C.−	−	1−	1	C.
4.4	−	±	1−	1	−	±	1−	C.
4.8	−	−	−	−	−	−	−	1
6.0	−	−	−	−	−	−	−	−
7.0	−	−	−	−	−	−	−	−
Distilled H_2O	−	−	−	−	−	−	−	−
NaCl M/14	−	−	−	−	−	−	−	−

TABLE VII.

Determination of the Acid Agglutination Zone of Staphylococcus aureus Grown at Room Temperature and at 37°C.

Readings made at the end of 24 hours.

Temperature of growth.	Agglutination.								
	pH of mixtures.								
	2.8	3.2	3.6	4.0	4.4	4.8	5.2	5.6	6.0
Room temperature.	1	−	−	−	−	−	−	−	−
37°C.	C.	C.	C.	3	C.	±	−	−	−

TABLE VIII.

Determination of the Acid Agglutination Zone of B. typhosus Grown at Room Temperature and at 37°C.

Readings made at the end of 24 hours.

Temperature of growth.	Agglutination.								
	pH of mixtures.							Controls.	
	2.8	3.2	3.6	4.0	4.4	4.8	5.2	Distilled H_2O	NaCl
Room temperature.	C.	C.	±	−	−	−	−	−	−
37°C.	C.	C.	C.	±	−	−	−	−	−

One experiment with *B. typhosus* showed no difference in the agglutination zones.

Table IX shows the effect of room temperature and 37°C. growth upon spontaneous agglutination when NaCl is the electrolyte employed. Further experiments with NaCl and K_2SO_4 show similar results, although in these no agglutination occurred in the room temperature growth except in the tubes containing saturated salt.

Table IX shows the effect of NaCl. It will be noted that there is no agglutination in the room temperature growth except at a concen-

TABLE IX.

Agglutination of Streptococcus (Strain 1372) in Various Concentrations of NaCl, When Grown at Room Temperature and at 37°C.

Readings made at the end of 24 hours.

Temperature of growth.	Agglutination.						
	Concentration of NaCl.						
	M/2	M/4	M/8	M/14	M/56	M/224	0
Room temperature.	—	—	—	—	3	—	—
37°C.	±	3+	C.—	C.	C.—	—	—

TABLE X.

Agglutination of Streptococcus (Strain ScB) in Various Concentrations of K_2SO_4, When Grown at Room Temperature and at 37°C.

Readings made at the end of 24 hours; reaction at room temperature.

Temperature of growth.	Agglutination.							
	Concentration of K_2SO_4.							
	Saturated.	M/4	M/12	M/36	M/108	M/327	M/981	0
Room temperature.	C.	—	—	—	—	—	—	—
37°C.	C.	C.	C.	C.	C.	C.	C.	—

tration of M/56, while the bacteria grown at 37°C. agglutinate in M/4 to M/56 concentrations. Table X shows the results when K_2SO_4 is employed as electrolyte.

Summing up the results of these experiments, we note that (1) streptococci grown at room temperature (17–23°C.) (*a*) are stable in all concentrations of NaCl ordinarily employed in the agglutination reaction and (*b*) possess an acid agglutination zone resembling

that of stable organisms in general; and (2) streptococci grown at 37°C. (*a*) are not stable in NaCl solutions, and (*b*) have either a widened acid agglutination zone or (*c*) if this zone is not widened, are unstable in the buffer solutions outside this pH zone for other reasons. This latter alternative would seem to be the correct one. Results presented further on would seem to show that the reason is a difference in the attracting forces.

In their recent work on the stability of bacterial suspensions (2, 3, 5–8), Northrop and De Kruif consider agglutination in terms of two forces: a repelling force which keeps the bacteria apart,—charge on the bacteria,—and an attracting or cohesive force. They have shown that the potential (charge on the bacteria) is the main factor in suspensions when the salt concentration is low (less than 0.01 N) and that the cohesive force plays the important part in higher salt concentrations. They have shown that, provided the cohesive force is unaffected, bacteria agglutinate when the potential lies between −15 and +15 millivolts; that is to say, when the cohesive force is large enough to overcome the repelling one. And they have shown that high concentrations of salts (over 0.01 N) reduce the cohesive force, and with this the critical potential agglutination zone.

Accordingly, in seeking for an explanation of the results above, it becomes necessary to look for differences of potential or cohesion, or both, in the two groups.

In considering the question of potential, differences must be investigated in suspensions containing salt in low concentration, to eliminate the cohesive factor. When high valency ions are employed, the effect on the potential can be obtained in high dilution.

Tables XI and XII show the effect upon agglutination and potential (Strain ScB) when thorium nitrate is used. The bacteria were first set up in varying dilutions to determine the point at which agglutination occurs (Table XI). As will be noted, this takes place from 5×10^{-5} to 6×10^{-6}.

Then, dilutions from 3×10^{-4} to 1.1×10^{-6} were taken and agglutination and potential determined. Table XII gives the results.

It will be seen that in very low concentration (Tubes 1 to 3) the bacteria retain their usual negative charge, and that in higher concentration (Tubes 4 and 5) the charge becomes positive. Complete agglutination occurs in Tube 3 only, P.D. −11 and −14 respectively; in Tubes 2 and 4 very doubtful flocculation occurs. This indicates

that the critical potential (agglutination) zone of the bacteria grown at room temperature lies somewhere between −17 and +17, and that of the 37°C. growth between −19 and +24. The difference between the two critical potential zones is thus very small, indicating that it is a very slight factor, if any. Determinations of potential of bacteria suspended in buffer solutions show similar minor differences.

TABLE XI.

Agglutination of Streptococci (Strain ScB) in Various Concentrations of Thorium Nitrate, When Grown at Room Temperature and at 37°C.

Readings made at the end of 24 hours; reaction at room temperature.

Temperature of growth.	Agglutination.					
	Molar concentration of $Th(NO_3)_4$.					
	5×10^{-4}	1.6×10^{-4}	5×10^{-5}	1.8×10^{-5}	6×10^{-6}	2×10^{-6}
Room temperature.	−	−	C.	C.	−	−
37°C.	−	−	C.	C.	C.	−

TABLE XII.

Agglutination and Potential of Streptococci (Strain ScB) in Various Concentrations of Thorium Nitrate, When Grown at Room Temperature and at 37°C.

Experiment at room temperature; agglutination readings made at the end of 24 hours.

Tube No.	Dilution of $Th(NO_3)_4$.	Bacteria grown at room temperature.		Bacteria grown at 37°C.	
		Agglutination.	Potential.	Agglutination.	Potential.
1	1.1×10^{-6}	−	−24	−	−23
2	2.2×10^{-6}	±	−17	±	−19
3	4.5×10^{-6}	C.	−11	C.	−14
4	9.0×10^{-6}	±	+17	±	+24
5	1.8×10^{-5}	−	+26	−	+29

Early in the investigation it was felt that the cohesive factor is the probable explanation of the differences between the room temperature and 37°C. growths.

In favor of this conjecture may be noted the following facts.

1. If complete agglutination be taken as the criterion for the acid agglutination zone (Tables II, IV, V, and VI), the zones of the two groups are practically the same. This observation is against a potential and in favor of a salt effect.

2. If we assume, from the results of the NaCl and K_2SO_4 experiments (Tables IX and X), that the cohesive force of the bacteria grown at the higher temperature is greater, the whole mechanism of the difference is explained. Reference to these tables shows that there is agglutination in high salt concentration in the 37°C. growth, and none in the other. Salt in these concentrations reduces the charge on the bacteria to limits well within the critical potential (agglutination) zone, but usually markedly depresses the cohesive force, and accordingly agglutination fails to occur (6). This, as noted, is true of the bacteria grown at room temperature; that is to say, cohesive force is reduced by the high salt concentration. If, however, the cohesive force is high or resistant to the salt effect in this zone of low potential, the bacteria will agglutinate; or, to put it another way, agglutination in this low potential zone, when high salt concentration is used, can only occur if the cohesive force remains high. This, as noted, occurs with the bacteria grown at 37°C.

3. The acid agglutination zone of staphylococci (Table VII) is virtually abolished by room temperature growth; that is to say, growth at this lower temperature either reduces cohesion or makes this force less resistant to salt effects; and additional lowering by the fairly high salt concentration of the buffers prevents agglutination in the acid zone in which the low potential usually leads to agglutination, and in which it leads to agglutination with the bacteria grown at 37°C.

On the basis of this reasoning, differences in the cohesive force of the two groups of bacteria would seem to be the probable explanation of the findings. Direct measurements of this force were now made and the results obtained confirm the supposition. As noted by Northrop and De Kruif (6), their method of determination of cohesive force is reasonably accurate; and in our hands, after the technique had been learned, the method, though not one of absolute precision, gave surprisingly parallel as well as reproducible results.

A brief review of the method devised by Northrop and De Kruif (6) may be given here.

Thick films of the bacteria are made on a heavy glass slide and on a cover-slip. These are allowed to dry and are then passed rapidly through a flame a few times to ensure adhesion to the glass. The slides are then placed in the dish of the du Noüy surface tension apparatus, film to film, in the solutions whose effect is to be tested, and after they have been left in contact 1 minute (no pressure is used) the force required to pull the films apart is measured.

Table XIII shows the effect of concentrated NaCl upon the cohesive force of the bacteria grown at room temperature and at 37°C. Measurements were made of the cohesive force, in distilled water, and in

concentrated NaCl (1.7 M), for Strain ScB. As will be noted, the cohesive force of the bacteria grown at room temperature is reduced by the concentrated salt; while that of the 37°C. growth is not only not reduced, but is actually slightly increased. Experiments with other films and with films of another strain (No. 1372) gave parallel, though not always such striking results.

From the results above, it seems reasonable to conclude that many strains of streptococci, when grown at the temperature of incubation employed as routine (37°C.), possess an attracting or cohesive force which is resistant to the depressing effects of the concentrations of salts commonly used as electrolytes, and on this account spontaneous

TABLE XIII.

Determination of the Cohesive Force of Smears of Bacteria Grown at Room Temperature and at 37°C.

Temperature of growth.	Solution in which smears were placed.	Readings on dial (torsion of wire).	Average.	Force required to separate 2 sq. cm. surface.
				mg.
Room temperature.	Distilled H_2O	15, 20, 19, 18	18	90
	NaCl 1.7 M	12, 13, 9, 10	11*	55
37°C.	Distilled H_2O	22, 20, 21	21	105
	NaCl 1.7 M	19, 30, 31, 30	27.5	137.5

* After immersion in NaCl solution, this was washed out with distilled water, and after resuspension in distilled water the cohesive force between the smears was reread and averaged 17.5 (87.5 mg.).

agglutination occurs.[6] Moreover, growth at a lower temperature in some way prevents this resistance, with resulting greater stability of suspensions.

When one approaches the problem of securing suspensions of streptococci that are satisfactory for use in agglutination reactions, the following facts must be noted.

[6] A number of workers with streptococci have overcome the difficulty with autoagglutinating strains by using low concentrations of NaCl as electrolytes. The probable explanation of their success lies in the fact that, with low salt concentration, the potential is high (5), and that then the repelling effect involved overcomes the high cohesive force and the bacteria become stable.

1. Many strains are habitually diffuse and always give satisfactory stable suspensions.

2. Many strains are habitually or intermittently granular, but by growth at room temperature may be made diffuse, and are then satisfactory to work with. Table XIV shows a specific agglutination test with a strain (No. 1372) belonging to this group. The electrolyte used was NaCl M/14 (0.425 per cent). It will be noted that the room temperature growth is stable in the high dilutions of serum and in the control, and that the agglutination titer is clear-cut; and that the 37°C. growth is unstable, complete agglutination occurring in all tubes, including the control.

TABLE XIV.

Specific Agglutination of Streptococci (Strain 1372) Grown at Room Temperature and at 37°C.

Electrolyte, NaCl M/14.

Temperature of growth.	Agglutination.									
	Serum dilution.								Controls.	
	0.1	0.01	0.005	0.0025	0.0012	0.0006	0.0003	0.00015	0	Distilled H_2O
Room temperature.	C.	C.	C.	C.	C.	C.—	2	—	—	—
37°C.	C.	C.	C.	C.	C.	C.	C.	C.	C.—	—

3. A few strains are always granular, even when grown at room temperature. A number of methods have been devised for coping with this difficulty. Northrop and De Kruif (12) have suspended washed bacteria in 0.001 N NaOH, and have found such a suspension stable when NaCl in low concentration (M/320) is used as electrolyte. Several observers, working with autoagglutinating bacteria, both streptococci and other organisms, have obtained stable suspensions by using very low concentrations of electrolytes; the probable explanation of their success has been noted above.

Table XV shows the application of these methods to a fairly granular strain. It will be noted here that the bacteria become stable in NaCl concentrations over 0.01 N, and that the bacteria treated with NaOH agglutinate more slowly than the untreated ones. It is probable

that the required concentration of electrolyte for stability will vary with different strains, becoming less as the strain is more granular.

Observations on the acid agglutination zones of streptococci (Tables II, IV, and V) show that the bacteria are more stable at pH 7 to 8. For this reason a very granular strain (Strain ScBF, scarlet fever) was tested against a specific scarlet fever streptococcus serum (No. 1372) in G.P.A. buffer, pH 7.0. Table XVI shows the results. It will be seen that agglutination is very slow in the control tube and quite marked in the tubes containing serum. Specific agglutination is therefore easily to be recognized on the basis of

TABLE XV.

Stability of Granular Streptococci (Strain ScB) in Various Concentrations of NaCl.

Condition of growth.	Time.	Agglutination. Concentration of NaCl.						
		3 M	1 M	0.33 M	0.11 M	0.037 M	0.012 M	0.004 M
	hrs.							
Bacteria suspended in distilled H_2O and added to NaCl solution.	1	−	−	−	−	−	−	−
	2	±	±	1−	1	±	−	−
	4	1	2	2+	3	+	−	−
	24	C.−	C.	C.	C.	±	−	−
Bacteria suspended in 0.001 N NaOH and added to NaCl solution.	1	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−
	4	−	±	±	−	−	−	−
	24	+	1	±	±	±	−	−

early readings, but would be impossible from a 24 hour reading. Similar results were obtained at pH 8.0. When NaCl (M/14) is used as electrolyte the agglutination in the control is too rapid for satisfactory comparative readings.

In view of the evidence that salts in low concentration favor stability, and in view of the observation (Table XIII) that there is an increase of the cohesive force of bacteria grown at 37°C. in the presence of salts in high concentration, washed bacteria were next tested for stability in G.P.A. buffer, pH 7.0, in lower concentration. The results appear in Table XVII, and show that greatest stability is obtained in low concentrations (less than M/100).

Stability was also sought for, but without success, by the use of NaCl, $BaCl_2$, and $CaCl_2$ in high concentration (saturated to M/10).

Finally (Table XVIII), the agglutination titer of specific serum, with the various methods above, was investigated. Granular growths, incubated at 37°C., of a streptococcus (Strain ScB) were

TABLE XVI.

Specific Agglutination of a Granular Streptococcus (Strain ScBF from Scarlet Fever) with Heterologous Serum (No. 1372) in Buffer Solution (G.P.A.) pH 7.0.

Bacteria grown at room temperature.

Time.	Agglutination.						
	Serum dilution.					Controls.	
	0.05	0.005	0.0025	0.0012	0.0006	No serum.	Distilled H_2O
hrs.							
1	1	1	1	1	1+	−	−
2	2+	3+	2+	2+	2+	±	−
3	3	C.−	3+	3+	3+	1	−
24	C.	C.	C.	C.	C.	3+	−

TABLE XVII.

Stability of Streptococci (Strain ScB) in Various Dilutions of G.P.A. Buffer, pH 7.0.

Time.	Agglutination.				
	Total salt concentration.				
	M/25	M/50	M/100	M/200	M/400
hrs.					
1	1	±	−	−	−
2	2	1	−	−	−
4	3	1+	±	−	−
24	C.	C.−	1	−	−

used, with a stable growth, made so by incubation at room temperature, of the same organism as the control. It will be noted that the titer of the serum is not appreciably affected (Table XVIII).

Taken together these experiments indicate that some strains of streptococci are always diffuse and give stable suspensions for specific agglutination; some are granular, but by growth at room temper-

ature may be made diffuse and will then yield satisfactory suspensions; and the few remaining refractory granular strains may be made less granular by growth at room temperature, and can then be tested satisfactorily with electrolytes in low concentration (especially when treated with NaOH), or else in buffer solutions of pH 7.0 to 8.0.

TABLE XVIII.

Specific Agglutination Titer of Antistreptococcus Serum with a Granular Strain (Strain ScB), with Various Electrolytes.

Readings made at the end of 24 hours.

Bacteria.	Electrolyte.	Agglutination.							
		Serum dilution.							Control.
		0.01	0.005	0.0025	0.0012	0.0006	0.0003	0.0001	
Granular streptococcus grown at 37°C.	NaCl M/14	C.	C.	C.	C.	C.	C.	C.	C.
	NaCl M/56	C.	C.	C.	C.	2	1	1	1
	NaCl M/56 + NaOH 0.001 N	C.	C.	C.	C.	2	±	±	±
	G.P.A. buffer. pH 7.0, M/25	3	C.	C.	C.−	2	1	1	1
	M/50	C.−	C.	C.	C.	2	2	1	1
	M/100	C.−	C.	C.	C.	2	1	±	±
Control, diffuse. Room temperature growth.	NaCl M/14	C.−	C.	C.	C.−	2	±	−	−
	Stock broth.	C.	C.	C.	C.	C.−	2	2	2

SUMMARY.

1. The spontaneous agglutination of streptococci has been studied.

2. This spontaneous agglutination would seem to be caused by the presence of a bacterial cohesive force higher than that usually found when bacteria are suspended in salt solutions of the concentration commonly employed as electrolyte in specific agglutination reactions.

3. Many granular autoagglutinating strains of streptococcus may be made diffuse by growth at room temperature (17–23°C.) and then lose their tendency to agglutinate spontaneously.

4. All factors that reduce cohesive force or that make the repelling force relatively greater than the cohesive force make for stable suspensions.

5. Methods for management of the specific agglutination of refractory autoagglutinating strains of streptococci have been presented.

The writer wishes to express his indebtedness to Dr. J. H. Northrop of The Rockefeller Institute for most valuable help in the course of the investigation.

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HOMOLOGOUS AND HETEROLOGOUS PROTECTION IN MICE VACCINATED WITH THE TWO TYPES OF MOUSE TYPHOID BACILLUS.

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Of the two epidemics of mouse typhoid described by Lynch[1] as occurring among the so called cancer stock at The Rockefeller Institute, the first, or 1918 epidemic was caused by a strain of mouse typhoid bacillus, Type I, while the second, or 1920 epidemic was caused by a Type II strain. In commenting upon this, Lynch says:

"The fact that the two strains of the paratyphoid-enteritidis group, differing immunologically from each other and both potentially capable of setting up severe epidemics among mice, were responsible for the epidemics separated from each other by 2 years, comes to have a special interest and may possess a particular significance in view of the vaccinations carried out in the period between the two epidemics. For superficially, at least, it appears that the inoculation of the killed cultures of the first bacillus shunted, as it were, that particular organism out of action while leaving the recruited population, both old and new, and the old even more than the new, subject to a second variety of the mouse typhoid bacillus."

Since the last epidemic in 1920, it has become a matter of routine to administer subcutaneously to the mice of this cancer stock a mixed saline vaccine containing both types of mouse typhoid bacillus, in an effort to maintain the immunity of the population at a level high enough to prevent another epidemic. No further outbreak of mouse typhoid, in anything like epidemic proportions, has occurred among these mice, although there is known to be a high fecal carrier incidence of M. T. I among them, and a slight carrier incidence of M. T. II also. Furthermore, in a single experiment in which twenty-five of these mice, the unvaccinated offspring of vaccinated mothers and themselves free of fecal contamination with either type of mouse

[1] Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15.

typhoid bacillus, were employed, they showed, when fed *per os* with M. T. II, a corrected mortality rate of only 12 per cent, as compared to a 64 per cent mortality among the controls. Practically no blood invasion occurred except in those mice which died, although among the controls blood invasion occurred at some time in 88 per cent.

Vaccination with the strain of M. T. II employed as a stock strain in our laboratory (the strain originally isolated by Amoss[2]) has been shown by Webster[3] to be effective in protecting mice against subsequent injection with living organisms of the homologous strain. Aside from the vaccination of the cancer stock with a mixed M. T. I and M. T. II vaccine, we had had no experience with vaccines made from Strain M. T. I except in the production of agglutinating sera in rabbits. For this reason some experiments were undertaken in the immunization of mice with vaccines made from each type of mouse typhoid bacillus, in an attempt to determine whether a heterologous as well as a homologous protection was conferred by each vaccine.

EXPERIMENTAL.

Experiment 1.—Six series of twenty-five mice each were assembled from the Rockefeller Institute breeding room, each mouse being placed in a separate battery jar. Certain of these mice had received previous vaccination, as follows:

Series A and F received subcutaneously in three successive weekly doses a monovalent M. T. I vaccine (1,000,000,000 organisms at each dose).

Series C and D received subcutaneously in three successive weekly doses a monovalent M. T. II vaccine (1,000,000,000 organisms at each dose).

The six series of mice were inoculated by means of a stomach tube 10 days after the last vaccination, as follows:

Series A (M. T. I vaccinated),	0.005	cc.	of	M. T.	I	broth	culture	*per os*.	
" B (controls),	0.005	"	"	"	I	"	"	"	"
" C (M. T. II vaccinated),	0.005	"	"	"	I	"	"	"	"
" D (" II "),	0.005	"	"	"	II	"	"	"	"
" E (controls),	0.005	"	"	"	II	"	"	"	"
" F (M. T. I vaccinated),	0.005	"	"	"	II	"	"	"	"

The outcome of this experiment was unexpected, as shown by mortality rates presented in Table I.

[2] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25.
[3] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71.

It appeared from these percentages that a definite immunity had been conferred by the M. T. II vaccine against its homologous strain, as compared with the natural immunity among the controls, but that the M. T. I vaccine, while it had apparently modified slightly the form of the mortality curve of those mice inoculated *per os* with the homologous strain, had afforded no more protection against this strain than was conferred by vaccination with the heterologous M. T. II, and had failed to confer any protection against the heterologous M. T. II when mice were subsequently inoculated with this organism *per os*.

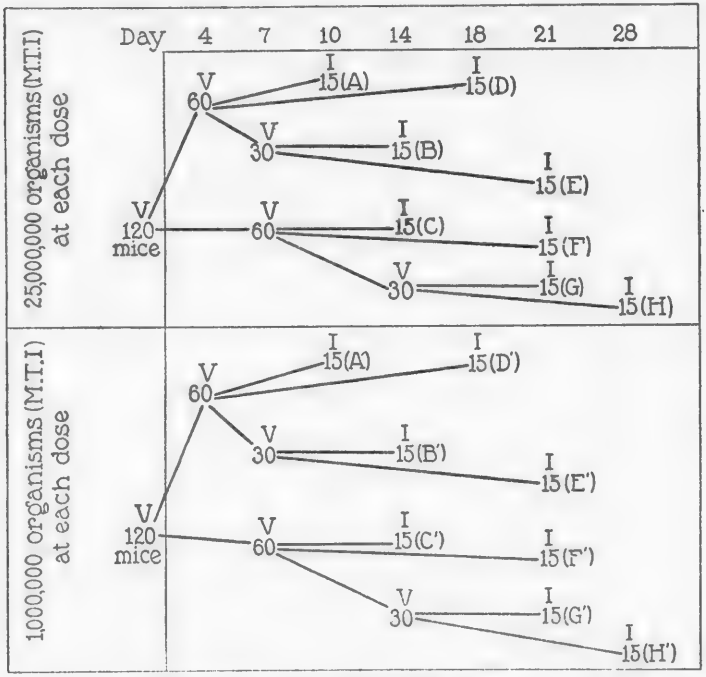
TABLE I.

Series.	Final mortality figures.	
	Total.	Corrected.*
	per cent	*per cent*
A	68	64
B	84	84
C	64	52
D	32	24
E	68	64
F	68	64

*Autopsies with negative blood and fecal cultures excluded.

Experiment 2.—A second attempt was made to obtain a positive protection against *per os* inoculation of M. T. I through the previous administration of an homologous vaccine. Owing to a temporary shortage of mice, smaller numbers had to be used and it was impossible to vaccinate a parallel series with M. T. II, to repeat the experiment in cross-protection. The vaccines employed in Experiment 1 had been made by washing off with saline the 24 hour growth from several Blake bottles and heating the emulsions thus obtained to 60°C. in the water bath for 1 hour. In Experiment 2 a somewhat different technique was employed. The M. T. I emulsion was twice washed in saline, after removal from the Blake bottles, and was then killed by heating to 56°C. in a water bath for 1 hour. It was hoped that the lower thermal death-point would kill the bacteria without too profoundly altering the bacterial protein. As a matter of fact, even after heating for 1 hour at 56°C., the vaccine contained a very few living organisms, two or three colonies developing when 0.1 cc. of the undiluted vaccine was smeared on a green dye plate. Thymol was added to the stock vaccine for a preservative as in Experiment 1.

In performing this experiment in vaccination with M. T. I, an effort was made to answer several questions: (*a*) Length of time after vaccination most favorable for *per os* inoculation with living organisms. In an effort to determine this, mice were inoculated 7 and 14 days after their last dose of vaccine. (*b*) Number of doses most effective for the production of immunity. Some mice were given two doses



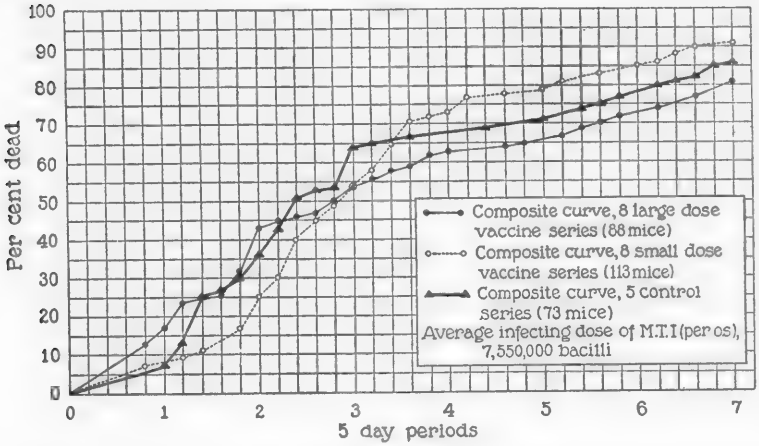
TEXT-FIG 1. Scheme used for the vaccination of mice with M. T. I when the size of the dose, the number of doses, and the interval between doses were varied. *V* indicates vaccinated; I, inoculated.

of vaccine, subcutaneously, and some three. (*c*) The size of the dose capable of producing immunity without proving toxic to the mouse. Half the mice received, subcutaneously, doses of 1,000,000 organisms each, and half received doses of 25,000,000 organisms each. Events proved that the smaller dose was not effective, and the larger dose too

toxic to be well borne. (*d*) Intensive as opposed to gradual vaccination. Half the mice received their doses of vaccine at intervals of 4 days, the other half at intervals of 1 week.

A scheme was worked out for the vaccination and inoculation of these mice, as shown in Text-fig. 1.

Composite curves, constructed from each series of mice, according to large or small dosage of vaccine, together with a composite control curve, are shown in Text-fig. 2. From these curves it will be seen that the mice receiving the smaller doses of vaccine showed, in the



TEXT-FIG 2. Composite total mortality curves constructed by averaging (*a*) all the small dose vaccine series (*A'* to *H'*, Text-fig. 1), (*b*) all the large dose vaccine series (*A* to *H*, Text-fig. 1), and (*c*) all the various control series.

beginning, a slight advantage over both the controls and the mice receiving larger doses of vaccine, but that this advantage was lost in the period between 15 and 18 days after inoculation. Thereafter the curves of the two vaccinated series ran an almost parallel course, with the small dose series some 10 per cent above the large dose series, and the control curve striking an almost exact average between the two, to the end of the experimental period.

The study of separate curves gives little or no evidence that one method of vaccine inoculation, as regards intervals between doses and

size of doses, was preferable to another, nor is there any clear-cut indication that a definite immunity against infection with M. T. I was conferred by any of the methods of vaccination employed. That the antigenic properties of the vaccine were not injured by heat was indicated by the fact that when injected intravenously into rabbits it was capable of calling forth the production of an excellent homologous

TABLE II.

Series.	Duration of experiment.	Day last death occurred.	No. of mice.	Final mortality figures.	
				Total.	Corrected.*
	days			*per cent*	*per cent*
A	35	29	13	86	86
A′	35	33	14	63	57
Controls.	35	34	15	93	93
B	35	31	12	81	75
B′	35	30	14	84	71
C	35	28	15	82	73
C′	35	33	15	82	73
Controls.	35	34	13	71	71
D	35	35	11	73	64
D′	35	31	14	100	93
Controls.	35	33	15	90	90
E	35	35	5	80	80
E′	35	30	15	100	80
F	35	10	8	100	100
F′	35	23	14	100	93
G	35	34	14	77	71
G′	35	32	14	100	86
Controls.	35	35	15	93	87
H	35	11	10	70	60
H′	35	25	13	93	77
Controls.	35	32	15	82	82

*Autopsies with negative blood and fecal cultures excluded.

Different sets of controls had to be used because separate groups of mice fell due for inoculation on different days (Text-fig. 1).

agglutinating serum. Mice, however, were unable to respond to the inoculations with the production of a demonstrable active immunity.

The final mortality figures for the different series are given in Table II.

The chief points of interest gained from this experiment were as follows: (*a*) The strain of M. T. I used in this laboratory is far more

toxic than the strain of M. T. II. It is not well borne, when administered subcutaneously to mice as a killed culture, even in doses as small as 25,000,000 organisms. In this experiment, each series originally included fifteen mice. At the time of their inoculation *per os* with living organisms, the original fifteen had diminished considerably, especially those mice that had received the larger doses, as may be seen from a survey of the numbers that finally came to inoculation (Table III).

The large dose appears to even greater disadvantage when further deductions are made for those mice that died at 24, 48, or 72 hours after the *per os* inoculation, and so had to be discarded as deaths probably due to accidents associated with the inoculation (Table II). There

TABLE III.

Series.	Large dose (25,000,000).	Series.	Small dose (1,000,000).
A	15 mice.	A′	15 mice.
B	14 "	B′	15 "
C	15 "	C′	15 "
D	13 "	D′	14 "
E	7 "	E′	15 "
F	9 "	F′	14 "
G	15 "	G′	14 "
H	11 "	H′	14 "

were ten such deaths among the various series of mice that had received the larger doses of vaccine (25,000,000 organisms), as against only three among the mice that had received the smaller doses (1,000,000 organisms). This may mean that the premature deaths were not wholly due to accidents of inoculation, but could be attributed, at least in part, to the lowered resistance of those animals that had received the larger doses of vaccine.

(*b*) This strain of M. T. I, originally isolated in our laboratory from a fatal case of mouse typhoid, is not an active antigen for the production of immunity in mice. A vaccine prepared with it, even when administered subcutaneously in two or three doses of 25,000,000 organisms each—doses large enough to prove toxic to some animals—is incapable of producing a definite immunity against subsequent

per os inoculation with living cultures of M. T. I. In this respect, as well as in its toxicity, it differs from the M. T. II strain isolated in this laboratory, which possesses more readily demonstrable antigenic properties, and is capable, when used as a vaccine, of conferring a definite immunity against the homologous strain, and possibly some slight cross-protection against the M. T. I strain also.

DISCUSSION.

The effect of vaccination with M. T. II has already been demonstrated in mice by Webster,[3] and by Topley and Wilson[4] who designate their organism *Bacillus enteritidis* (*aertrycke*). Nichols and Stimmel[5] have recently described successful vaccination experiments in guinea pigs with a strain of *Bacillus aertrycke* (mutton) derived from a spontaneous epidemic among these animals, an organism which Webster[6] found to be closely related to our M. T. II strain. In the present series of experiments also, M. T. II vaccine has been found fairly effective in protecting mice against subsequent *per os* inoculation with the homologous strain. M. T. I vaccine, on the other hand, has failed to produce in mice, under the experimental conditions that existed here, any clearly demonstrable immunity against its homologous strain when living organisms of this strain were later administered *per os*. The two types of mouse typhoid bacillus appear to differ radically both in their toxic properties and in their ability to act as antigens in mice.

Under these circumstances, a question naturally arises as to the part played by the polyvalent mouse typhoid vaccine in preventing an epidemic of typhoid, due to the Type I bacillus, among the cancer stock of The Rockefeller Institute. Since fecal carriers of M. T. I are fairly numerous among these mice, they are constantly exposed to infection from this source, yet no epidemic due to M. T. I has occurred among them since 1918. Since the M. T. II outbreak in 1920, a certain level of immunity to M. T. II has been artificially maintained through vaccination of all young mice. The experiment in cross-

[4] Topley, W. W. C., and Wilson, G. S., *J. Hyg.*, 1923, xxi, 243.
[5] Nichols, H. J., and Stimmel, C. O., *J. Exp. Med.*, 1923, xxxviii, 283.
[6] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

protection indicates that some slight cross-protection may be conferred against *per os* inoculation of M. T. I by the administration of M. T. II vaccine, and with the relatively much smaller doses of living organisms ingested by mice under normal living conditions, with carriers present, this protection might be still more effective. It is, of course, possible that the general level of resistance of a mouse population to infection with mouse typhoid bacillus Type I might be raised through long exposure to small doses of living organisms excreted by carriers and ingested with food, even though a killed vaccine prepared from this strain is ineffective in producing immunity on subcutaneous inoculation. It seems reasonable to assume that M. T. II infection in this stock has been kept down by the homologous strain included in the vaccine administered to them, but the freedom of the animals from infection with M. T. I, in the presence of numerous carriers of this organism, can scarcely be attributed to the effects of the Type I strain included in the vaccine in view of the negative results obtained in the vaccination experiments here recorded.

SUMMARY.

A saline vaccine prepared from our stock strain of M. T. II produces in mice definite protection against the homologous strain, and perhaps some slight cross-protection against M. T. I also. Two vaccines prepared with our stock strain of M. T. I failed to produce any definite protection in mice even against the homologous strain, nor was any cross-protection afforded against M. T. II.

THE OCCURRENCE OF PEROXIDE IN CULTURES OF PNEUMOCOCCUS.

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McLeod and Govenlock (1) in 1921 reported the occurrence in pneumococcus cultures of a heat-labile substance which was inhibitory to the further growth of this and other organisms. The name bactericidin was first suggested by these authors. Further work by McLeod and Gordon (2, 3) indicates that this substance is hydrogen peroxide, and that it may occur in cultures of organisms other than pneumococcus, as *Bacillus bulgaricus*, *Bacillus acidophilus*, and certain coccal and sarcinal forms. In a recent communication (4) these authors present a scheme of classification of bacteria based upon their properties of catalase production and sensitiveness to hydrogen peroxide.

The growth-inhibitory substance of bacterial origin was found by the English investigators to be sensitive to the action of heat (85°C.), and of catalase; the gas evolved on adding catalase to a culture concentrate proved to be oxygen. From these and other chemical reactions they conclude that this unstable bactericidal product of growth which develops particularly in cultures of pneumococcus, is hydrogen peroxide rather than some organic peroxide. It does not occur in cultures deprived of oxygen or in those which contain abundant catalase. McLeod and Gordon further ascribe to the action of this substance the changes in blood pigment effected by growth of pneumococcus. They conclude that the early death of pneumococci in cultures is usually due to the accumulation of an excess of hydrogen peroxide.

The present studies on bacterial peroxide, reported in this and subsequent papers, deal with the occurrence of this substance in cultures of pneumococci, its effect upon bacterial growth, and its relation to other phenomena of cell activity. The present paper is concerned with (1) the factors influencing the occurrence of peroxide in cultures of pneumococcus, (2) factors influencing the stability of

peroxide in cultures of pneumococcus, and (3) the occurrence of peroxide in cultures of pneumococcus, *Streptococcus viridans*, *Streptococcus mucosus*, *Streptococcus hæmolyticus*, and *Staphylococcus aureus*.

EXPERIMENTAL.

1. Factors Influencing the Occurrence of Peroxide in Cultures of Pneumococcus.

Exposure to Atmospheric Oxygen.—From preliminary experiments it became evident that the rapidity with which peroxide is formed in broth cultures of pneumococcus is dependent, in part at least, upon the depth of the column of culture fluid, and the extent of the surface area in direct contact with air.[1] In order to demonstrate this fact further, the following experiment was performed.

Experiment 1.—150 cc. of plain broth were placed in a 300 cc. Florence flask and inoculated with 0.1 cc. of an 8 hour plain broth culture of Pneumococcus Type II. At the same time 50 cc. of the same lot of broth in a 500 cc. Erlenmeyer flask were similarly inoculated. Both flasks were incubated at 37°C. Tests for peroxide were made after 18 and 24 hour periods of incubation. Autolysis was noted in both cultures at the 24 hour observation. The results are given in Table I.

Experiment 1 shows that peroxide is demonstrable early in a broth culture of pneumococcus which has a large surface of shallow culture fluid directly exposed to air. On the other hand, if pneumococci are grown under conditions which allow only a small surface area of fluid to be in contact with air, that is if the culture flask has a diameter such that the column of fluid is deep, rather than shallow, peroxide formation does not occur early.

That oxygen must be available before peroxide formation can occur, is further demonstrated by Experiment 2.

[1] The test for peroxide used in this work is as follows: 0.5 cc. of the fluid to be tested is placed in a small test-tube. A piece of fresh unheated potato, 1 by 0.5 cm., is utilized as the peroxidase. This is immersed in the fluid and 2 or 3 drops of freshly prepared saturated glacial acetic acid solution of benzidine is added. The color reaction indicating the presence of peroxide varies from a faint light blue, confined to the plant tissue, to an intense dark blue, discoloring both the potato and the fluid. The reaction is prompt, and the color fades rapidly, so that readings should be made at the time the test is performed.

Experiment 2.—Two 250 cc. Erlenmeyer flasks, each containing 50 cc. of broth were placed in boiling water for 30 minutes, and then rapidly cooled without shaking. Both flasks were then seeded with 0.5 cc. of an 8 hour plain broth culture of pneumococcus. One flask was placed immediately in an anaerobic jar, while with the other no attempt was made to exclude oxygen during incubation. After 36 hours at 37°C., at which time the cultures were undergoing autolysis, the contents of both flasks were passed through separate Berkefeld filters. The filtrates were then tested for peroxide. The results are shown in Table II.

TABLE I.

Effect of Surface Exposure of Broth Culture of Pneumococcus upon Peroxide Formation.

Type of container and amount of culture fluid.	Inoculum. 8 hr. broth culture of Pneumococcus Type II.	Peroxide test after incubation.	
		18 hrs.	24 hrs.*
	cc.		
300 cc. Florence flask containing 150 cc. of broth.	0.1	−	−
500 " Erlenmeyer " " 50 " " "	0.1	+	+

* Autolysis present in both cultures.

TABLE II.

Effect of Absence of Free Oxygen upon Peroxide Formation by Pneumococcus.

Type of container and amount of culture fluid.	Inoculum. 8 hr. broth culture of Pneumococcus Type II.	Peroxide test after 36 hrs. incubation at 37°C.	
		Anaerobic jar.	Atmospheric oxygen.
	cc.		
250 cc. Erlenmeyer flask containing 50 cc. of broth.	0.5	−	
250 " " " " 50 " " "	0.5		+

In Experiment 2 the growth conditions of both cultures were identical, with the exception that one was deprived of oxygen, while the other grew at atmospheric tension. In the culture grown under anaerobic conditions no peroxide was demonstrable. In the aerobic culture a strongly positive test for peroxide was obtained.

An effort has been made to investigate further the occurrence and the time of appearance of peroxide with respect to the oxygen exposure

of the medium. In Experiments 3, 4, and 5, the ratio of surface area to total volume of culture fluid has been used as a measure of the access of oxygen to the medium.

The Time of Appearance of Peroxide When the Surface Area-Volume Ratio Is Increased (0.8).—The following experiment was planned to show the time of appearance of peroxide in a pneumococcus culture grown under conditions favorable for peroxide production. During growth of the culture, fractions were examined at intervals, by colony count and pH determinations, to ascertain at what phase of the growth peroxide first appeared.

TABLE III.

Time of Appearance of Peroxide in Broth Culture with a Large Surface Area-Volume Ratio (0.8).

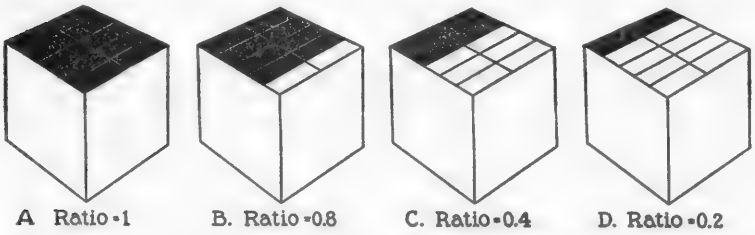
Time after seeding Pneumococcus Type II.	No. of colonies per cc. of culture.	pH of substrates.	Presence of peroxide in culture filtrate.
hrs.			
0	3,960	7.7	0
4	273,000	7.7	0
7	8,600,000	7.7	0
10	50,500,000	7.4	+
13	450,000,000	7.3	++
16	179,000,000	7.3	+++

Experiment 3.—In a 4 liter Erlenmeyer flask, 450 cc. of broth were placed. The medium formed a shallow layer about 2 cm. in depth. In this experiment the ratio of surface area to total volume of the medium was 0.8 (Text-fig. 1). This was seeded with 0.5 cc. of a 6 hour culture of Pneumococcus Type II. At intervals during incubation samples of the culture were removed, and colony counts made by plating measured amounts in glucose agar. Portions of these same samples were passed through Berkefeld filters, and pH determinations were made on the filtrates by the colorimetric method. Each sample of culture fluid was tested immediately after filtration for the presence of peroxide. The results are given in Table III.

From the colony counts and pH determinations recorded in Table III, it is seen that under the cultural conditions of this experiment the usual period of bacterial lag was avoided. Immediate multiplication of the organisms occurred, and growth progressed steadily until about the 13th hour, when autolysis began. Peroxide was

absent in the fractions of the culture removed before and at the 7th hour of incubation. All fractions removed after the 10th hour contained peroxide. The formation of peroxide, measured by the intensity of the color change in the benzidine reagent, reached its maximum at the 16th hour of incubation. Under these favorable conditions of growth and oxygen tension, peroxide was demonstrable in the culture filtrate between the 7th and 10th hours of incubation; that is, at a time when the culture was in the logarithmic phase of growth, as indicated by the colony count and by the changing pH.

Time of Appearance of Peroxide When the Surface Area-Volume Ratios Are Diminished (0.4 and 0.2).—As pointed out, for the rapid



TEXT-FIG. 1. Diagram to illustrate $\frac{\text{Area}}{\text{Volume}}$ ratios in Experiments 3, 4, and 5. The black portion represents the surface area exposed to air. *A* represents a hypothetical volume of fluid, the exposed surface area of which is 1 sq. cm. per cc. *B*, *C*, and *D*, similarly, represent volumes, the exposed surface areas of which are respectively 0.8, 0.4, and 0.2 sq. cm. per cc. *B*, *C*, and *D* represent the actual exposed surface area to volume relations which were employed in Experiments 3, 4, and 5, respectively.

appearance of peroxide in aerobic cultures of pneumococcus, the free access of oxygen is essential. Accordingly, the medium should be exposed in a shallow layer with a large surface area in contact with air. In the absence of this condition, peroxide may occur, but its appearance is markedly delayed.

Experiment 4.—A 300 cc. Florence flask containing 150 cc. of plain broth was inoculated with 0.2 cc. of a 6 hour broth culture of Pneumococcus Type II. The use of a relatively large inoculum from a rapidly growing pneumococcus culture insured immediate growth. The flask was incubated at 37°C. and, at the intervals noted in Table IV, 1 cc. portions of the culture were removed and tested

for peroxide. Autolysis of the culture was evident after 18 hours incubation. In this experiment the ratio of surface area to total volume of the medium was 0.4 (Text-fig. 1). The results of the peroxide tests are given in Table IV.

Analysis of the results presented in Table IV indicates that when pneumococcus is grown in broth in which the ratio of surface area to total volume is such that 0.4 sq. cm. per cc. of the medium is exposed to air, the presence of peroxide is demonstrable only after a considerable period of time (between 2 and 3 days in Experiment 4).

If pneumococcus is grown aerobically in a flask still larger than that used in Experiment 4, thus allowing a greater surface of fluid to be exposed to air, but in a volume so great that the surface area of the fluid is, as compared with its total volume, actually much

TABLE IV.

Time of Appearance of Peroxide in Broth Culture with the Average Surface Area-Volume Ratio (0.4).

Amount of broth and type of container used.	Inoculum.	Time after seeding.	Peroxide test.
		hrs.	
300 cc. Florence flask containing 150 cc. of broth.	0.2 cc. of a 6 hr. broth culture of Pneumococcus Type II.	12	0
		24	0
		48	0
		74	+
		98	++
		122	+++

less than in Experiment 4, peroxide should not become demonstrable until an even later time. This point was investigated in Experiment 5.

Experiment 5.—A 3 liter Florence flask containing 1,500 cc. of plain broth was seeded with 5 cc. of an 8 hour culture of Pneumococcus Type II and placed in the incubator at 37°C. At the intervals noted in Table V, 1 cc. portions of the culture were removed and tested for peroxide. In this experiment the ratio of surface area to total volume of the medium was 0.2 (Text-fig. 1). The results are recorded in Table V.

In Experiment 5, the ratio of surface area to volume of culture fluid was less than in Experiment 4; in Experiment 4 the actual surface area per cubic centimeter of culture fluid that was exposed to air was twice as great as that in Experiment 5. The conditions,

therefore, in the two experiments were such as so to alter the ratio of surface area to volume that proportionately less of the total medium was in direct contact with air in Experiment 5 than in Experiment 4. Under these conditions peroxide was not demonstrable until after the 17th day of incubation, at a time when marked autolysis was present. Reference to Text-fig. 1, in which is illustrated the actual surface area to volume ratios used in Experiments 3, 4, and 5, makes it evident that the larger this ratio the earlier is the time of appearance of peroxide in the culture fluids.

In Experiments 4 and 5, in which the time of appearance of peroxide was delayed, it does not seem likely that peroxide actually was not formed. Under these conditions it seems more reasonable to assume

TABLE V.

Time of Appearance of Peroxide in Broth Culture with a Small Surface Area-Volume Ratio (0.2).

Amount of broth and type of container used.	Inoculum.	Time after seeding.*	Peroxide test.
		days	
3,000 cc. Florence flask containing 1,500 cc. of broth.	5 cc. of an 8 hr. broth culture of Pneumococcus Type II.	6	0
		14	0
		17	0
		19	+

* Between the 6th and 14th days tests were performed daily.

that it is produced only at the surface of the culture fluid and that by diffusion it is either diluted in the total volume beyond a point which gives a positive test, or that, as it diffuses into the medium it is destroyed by other substances present in the culture fluid.

The Effect of Catalase on Peroxide Accumulation.—Hydrogen peroxide is rapidly decomposed in the presence of the catalase of plant tissue. It seemed of interest, then, to determine whether peroxide is demonstrable in a broth culture of pneumococcus grown in the presence of fresh plant tissue (5).

Experiment 6.—A 250 cc. Erlenmeyer flask containing 50 cc. of plain broth and pieces of sterile, unheated potato,[2] was placed in the incubator at 37°C. for

[2] Sterile cylindrical pieces of raw potato, 1 by 2.5 cm., were obtained by the technique described in a previous communication (Thjötta, T., and Avery, O. T., *J. Exp. Med.*, 1921, xxxiv, 455).

24 hours to insure sterility. A flask of the same size and containing the same amount of broth, without vegetable tissue, served as control. Both flasks were inoculated with 0.5 cc. of an 8 hour broth culture of Pneumococcus Type II. After 18 hours incubation, both cultures were filtered through Berkefeld candles, and the filtrates were tested for the presence of peroxide. The filtrate of the culture in plain broth alone gave a strongly positive peroxide reaction. The filtrate of the culture containing unheated potato gave a negative reaction.

In Experiment 6 conditions of oxygen tension favorable for peroxide formation were provided, but catalase, in the form of unheated vegetable tissue, was present in one of the two broth cultures. In the culture containing catalase, peroxide either was not formed, or was so rapidly destroyed that it did not accumulate in quantities sufficient to give a positive test. In the control broth culture, which contained no catalase, peroxide was readily demonstrable.

The results of this and other experiments indicate that the presence of a catalytic agent in the medium serves to prevent the accumulation of peroxide in the culture fluid. Since peroxides of this nature have been shown to be inhibitory and even bactericidal, it seems not unlikely that the stimulating influence of unheated plant tissue upon the growth of pneumococcus may be attributable, in part at least, to the decomposition by the vegetable oxidases of these deleterious products of bacterial metabolism.

2. *Factors Influencing the Stability of Peroxide in Cultures of Pneumococcus.*

The Persistence of Peroxide in Broth Cultures.—In order to ascertain the length of time peroxide persists, when once formed, in pneumococcus cultures, several strains were tested daily for the presence of this substance in the culture fluid.

Experiment 7.—8 hour broth cultures of seven representative strains[3] of pneumococcus were each subcultured into 15 cc. quantities of plain broth. The broth was placed in Erlenmeyer flasks of such size that the culture fluid reached a depth of about 1 cm. At frequent intervals during incubation, 0.5 cc. samples of each culture were tested for the presence of peroxide. It was found that peroxide was present in every instance after 18 hours at 37°C. After 6 days

[3] Four strains of Pneumococcus Type I, one strain of Pneumococcus Type II, one strain of Pneumococcus Type III, and one strain of Pneumococcus Group IV.

incubation at 37°C., the peroxide reaction had become faint, though it was still present in all cultures. At the end of the 12th day of incubation, it had disappeared from all cultures save one.

Under the conditions of Experiment 7, peroxide was present at the 18th hour of incubation in all of the seven pneumococcus cultures tested. The color reaction denoting the presence of peroxide showed a tendency to diminish in intensity after the cultures had been incubated for about 6 days. During the period between the 6th and 12th days of incubation, the test for peroxide became negative in all but one of the cultures. In the latter culture (Type I) peroxide was still present at the end of the 12th day of incubation.

The Influence of Hydrogen Ion Concentration, Temperature, and Oxygen Tension on the Stability of Peroxide.—Early in the study of the occurrence of peroxide in culture filtrates of pneumococcus, it became evident that, under certain conditions, this substance was quite labile, and that in this respect it behaved in a manner similar to commercial hydrogen peroxide. It seemed of interest, therefore, to determine the influence of certain factors, as temperature, hydrogen ion concentration, and exposure to air, upon the stability of this substance in culture fluids.

Experiment 8.—A culture of Pneumococcus Type I was grown in 450 cc. of plain broth in a 2 liter Erlenmeyer flask. At the end of 36 hours incubation 400 cc. of the culture were removed and passed through a Berkefeld N filter. The filtrate was divided into three parts: one fraction was adjusted to pH 5.1; the second to pH 7.1; and the third to pH 7.8. The three fractions gave a strongly positive test for peroxide. Tubes containing the filtrate at these three different hydrogen ion concentrations were stored at 4° and 37°C. in the dark. Half of the tubes were stored without seal; air was excluded from the remaining tubes by a vaseline seal 1 cm. deep. Tests for the persistence of peroxide under these different conditions were made at 4, 7, 10, and 14 day intervals. The results of the experiment are given in Table VI.

It is seen from the results given in Table VI, that peroxide disappears relatively rapidly from pneumococcus culture filtrates when the latter are stored in the dark at 37°C. In the absence of light at 4°C., however, the substance is relatively stable, regardless of whether the reaction of the filtrate is neutral, acid, or alkaline. On the other hand, at 37°C. in the dark, peroxide is less stable in culture

filtrates of pH 7.8 than in those of reactions of pH 7.1 or 5.1. The exclusion of air by means of a vaseline seal favors the disappearance of peroxide from pneumococcus culture filtrates. This latter phenomenon is due, possibly, to the action of certain reducing substances in the filtrate.

TABLE VI.

Influence of Temperature, pH, and Exclusion of Air upon the Stability of Peroxide Formed in Pneumococcus Culture Filtrates.

Pneumococcus culture filtrate.		Peroxide tests upon filtrate stored in the dark at 4°C.				Peroxide tests upon filtrate stored in the dark at 37°C.			
pH	Vaseline seal.	4 days.	7 days.	10 days.	14 days.	4 days.	7 days.	10 days.	14 days.
5.1	+	+	+	+	+	+	0	0	0
5.1	0	+	+	+	+	+	+	0	0
7.1	+	+	+	+	+	+	0	0	0
7.1	0	+	+	+	+	+	+	0	0
7.8	+	+	+	+	+	0	0	0	0
7.8	0	+	+	+	+	0	0	0	0

Influence of Heat on the Stability of Peroxide.—

Experiment 9.—A 250 cc. Erlenmeyer flask containing 50 cc. of broth was seeded with a 6 hour broth culture of Pneumococcus Type II. After 27 hours at 37°C. the culture was passed through a Berkefeld filter. The filtrate gave a positive peroxide reaction. Two fractions of the filtrate were placed in boiling water, one for 5 minutes, and the other for 15 minutes. A third fraction was autoclaved at 16 pounds pressure for 10 minutes. The filtrate which had been subjected to 100°C. for 5 minutes showed a faintly positive reaction. The portions of filtrate which had been exposed to 100°C. for 15 minutes, and 120°C. for 10 minutes, gave a negative test.

Peroxide, in the concentrations present in the culture filtrate tested in this experiment, was partially destroyed by exposure to 100°C. for 5 minutes, and completely destroyed by exposure to 100°C. for 15 minutes, and 120°C. for 10 minutes. McLeod and Gordon (2) found that the hydrogen peroxide formed in bacterial cultures is sensitive to heat, decomposing rapidly at temperatures above 85°C.

3. The Occurrence of Peroxide in Cultures of Pneumococcus, Streptococcus viridans, Streptococcus mucosus, Streptococcus hæmolyticus, and Staphylococcus aureus; Time of Appearance and Disappearance.

In order to extend these observations to representatives of the commoner pathogenic Gram-positive cocci, the following organisms were tested under conditions favorable for the production and accumulation of peroxide: 7 strains of pneumococcus,[4] including representatives of Types I, II, and III, and Group IV; 6 strains of non-hemolytic streptococci;[5] 3 strains of *Streptococcus mucosus;*[6] 2 strains of *Staphylococcus aureus;*[7] and 23 strains of *Streptococcus hæmolyticus.*[8] 14 of the 23 strains of hemolytic streptococci studied were of the human type, and 9 were isolated from dairy sources.

Experiment 10.—All of the 41 strains of Gram-positive cocci studied were removed from media containing blood or other catalytic substance by at least three subcultures in plain broth. 0.1 cc. of an 8 hour broth culture of each strain was seeded into a 50 cc. Erlenmeyer flask containing 25 cc. of plain broth. After 18 hours at 37°C. all strains were subcultured upon blood agar plates in order to verify the purity of the cultures. Tests for peroxide were made after 18 hours at 37°C. and thereafter at intervals of about 24 hours for a period of 12 days.

Each strain of pneumococcus and *Streptococcus viridans* studied gave the peroxide test at the end of 18 hours incubation. Of the three strains of *Streptococcus mucosus* only one gave a positive reaction at any time and in this instance the reaction appeared first on the 3rd day. The two strains of *Staphylococcus aureus* gave negative tests throughout the period of observation. *Staphylococcus aureus* possesses an active catalase, and to this fact may be attributed the absence of peroxide. Of the twenty-three strains of *Streptococcus hæmolyticus*, eight, or 35 per cent, gave no peroxide reaction. Per-

[4] Pneumococcus: strains, J.15, J.17, J.18, J.32, J.33, D.39, and I/191/11.

[5] Non-hemolytic streptococcus: strains, A.179, A.148, B.26, B.38, D.38, and E.1.

[6] *Streptococcus mucosus:* strains, M.I, M.II, and Ching.

[7] *Staphylococcus aureus:* strains, Staph. O. and Staph. R.

[8] *Streptococcus hæmolyticus:* strains, 37, 127, 66, 137, 55, 60, 19, 89, 21, 84, 23, 60 A, 32, 273, W.G., C.57, Phila., M.93, Man., R. milk, Anc., S.F., M.43.

oxide was demonstrated in the culture fluid of the remaining fifteen strains (65 per cent). The average time of appearance of peroxide in cultures of *Streptococcus hæmolyticus* was between the 3rd and 5th days of incubation. Two of these strains gave a positive reaction before the 86th hour of incubation, and four gave it only after the 110th hour. Of the total number of hemolytic streptococci studied, it is interesting to observe that twelve, or 84 per cent, of the fourteen strains of the human type showed the formation of peroxide, while six, or 66 per cent, of the nine strains from dairy sources gave negative results.

SUMMARY.

1. Conditions which favor the formation and accumulation of peroxide in broth cultures of pneumococcus are free access of air, and the absence of catalase, peroxidase, and other catalysts capable of decomposing this compound. Under these favorable conditions peroxide becomes demonstrable in the culture fluid during the logarithmic phase of growth and persists for a period of at least 6 to 12 days.

2. In the absence of these favorable conditions the formation of peroxide is inhibited. In a culture with deficient oxygen exposure the accumulation of peroxide is delayed; when anaerobic conditions are maintained the substance is not formed. In the presence of active catalysts, peroxide does not accumulate in the medium in quantities sufficient to give a positive reaction. The accumulation of peroxide in pneumococcus cultures is dependent upon the balance between the amount produced by the microorganisms and the amount destroyed by substances in the medium.

3. The peroxide formed in pneumococcus cultures is unstable. It gradually disappears during prolonged incubation at 37°C.; it is less stable in alkaline than in neutral or acid media. It is destroyed in culture filtrates exposed to the temperature of boiling water for 15 minutes, and to that of steam under pressure (15 pounds) for 10 minutes.

4. Peroxide formation occurred early in broth cultures of the seven strains of pneumococcus and of the six strains of non-hemolytic streptococci studied. Fifteen of twenty-three strains of *Streptococcus hæmolyticus* and one of three strains of *Streptococcus mucosus* formed

peroxide. In the positively reacting cultures of *Streptococcus hæmolyticus* and *Streptococcus mucosus* the presence of peroxide was not demonstrable until the 3rd to 5th day of incubation. Peroxide could not be detected at any time during growth of the two strains of *Staphylococcus aureus*.

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STUDIES ON BACTERIAL NUTRITION.

V. The Effect of Plant Tissue upon the Growth of Anaerobic Bacilli.

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INTRODUCTION.

In previous papers of this series it has been shown that unheated vegetable tissue, when added to buffered broth, not only makes possible, in the absence of blood, the cultivation of the so called hemoglobinophilic organisms (1), but also greatly favors the growth of other entirely unrelated bacteria, as for example certain Gram-positive cocci (2).

In the case of the pneumococcus, a seeding so minute as to cause in dextrose broth a period of bacterial lag extending over 18 hours will amply suffice to induce prompt growth in plain broth to which pieces of sterile unheated potato have been added. The presence of vegetable tissue causes other changes in the configuration of the growth curve. There is a prolongation of both the stationary phase and phase of decline of the pneumococcus culture. Moreover, in plant tissue medium the zone of pH within which growth can be initiated is extended beyond the acid and alkaline limits of the range in ordinary bouillon. A study of the behavior of other Gram-positive organisms shows that the growth of *Streptococcus viridans* and *Streptococcus hæmolyticus* is greatly accelerated in vegetable tissue media.

The present communication deals with the effect of plant tissue upon the growth of anaerobic organisms. Since the exclusion of air is the prime requisite for growth of obligate anaerobes in ordinary media, it seemed of interest to determine the effect of heated and unheated vegetable tissue upon this cultural requirement.

Tizzoni, Cattani, and Boquis (3) in 1889 demonstrated that, by the use of coagulated blood medium, the tetanus bacillus could be grown in the presence of air. Kedrowski (4), employing a different method, was also able to grow anaerobes

in the presence of oxygen. An agar slant was seeded with an aerobic organism. After growth had occurred the culture was killed by chloroform vapor, and a small amount of broth added. Anaerobes seeded into this medium grew even when no precaution was taken to exclude air. Theobald Smith (5) in 1890 first grew anaerobic bacteria in media containing sterile animal tissue and found that under these conditions the rigid exclusion of air was not necessary. These facts were confirmed in 1905 by Tarozzi (6) and by Wrzosek (7).

Unheated plant tissue was first employed in bacteriology by Ori (8). This investigator and Tarozzi (9) found that the growth of anaerobes was favored by the presence of vegetable tissue in the medium. Wrzosek (10) likewise used vegetable tissue in the cultivation of this group of organisms. In his hands heated plant tissue was more effective than fresh tissue, and by the addition of it to ordinary media, he was able to grow anaerobes for one subculture in the presence of air. Wrzosek found that the "substance" contained in the plant tissue which facilitated aerobic growth was not destroyed by desiccation and was relatively thermostable, resisting freezing and a temperature of 140°C. It was not affected by light, but deteriorated rapidly in the presence of air. He also used charcoal, zinc, iron, etc., in media for the cultivation of anaerobes, and concluded that these substances, as well as animal and plant tissue, favored growth of anaerobic bacteria by virtue of the reducing substances they contained. Kitt (11) also found that he could induce the rauschbrand bacillus to grow under aerobic conditions by seeding large quantities of bouillon with a large inoculum.

In the present work the anaerobic organisms studied were seeded into media exposed to air and containing heated and unheated plant tissue. In each instance two or more inocula, of different size, were used; and when growth occurred subcultures were made into media of the same type. Cultivation was continued, when possible, until the organisms had been grown in at least three subcultures under aerobic conditions.

EXPERIMENTAL.

Media.—The medium was freshly prepared. Pieces of potato[1] weighing approximately 0.5 gm. were placed in sterile tubes 15 by 1.5 cm. and 5 cc. of phosphate broth were added. Air was not excluded during incubation nor was any attempt made to exhaust the medium of air prior to inoculation. Three varieties of this medium were employed. The first consisted of plain broth containing 0.5 gm. of sterile unheated potato; the second, of broth containing 0.5 gm. of potato which had been boiled for 5 minutes in normal salt solution; the third, of

[1] The potato was obtained by the technique described in previous papers of this series.

broth containing 0.5 gm. of potato which had been autoclaved for 45 minutes at 120°C. Uninoculated samples of each variety of medium were incubated at 37°C. for 48 hours as controls.

Organisms.—The organisms used in the experiment were *Bacterium pneumosintes*, *B. histolyticus*, *B. chauvœi*, *B. aerofœditis*, and *B. œdematiens*.[2] All five of these anaerobic organisms grew promptly and abundantly when seeded into potato broth covered by a vaseline seal. In the case of *B. aerofœtidis* and of *B. œdematiens* gas was formed in potato broth.

Procedure.—The first series of cultures were made by inoculating the three types of "aerobic" media with varying amounts of 8 hour potato broth anaerobic cultures of the test organisms. Since it was found that the results varied considerably with the size of the inocula employed, several tubes were inoculated at each subculture with varying amounts of the parent culture. The amounts used for seeding were (1) the quantity on a platinum needle which had been thrust the depth of 1 cm. in the parent cultures, (2) one 2 mm. loop, (3) 0.05 cc., and (4) 0.1 cc. When growth occurred in the "aerobic" culture a second transfer was made to medium of the same type; *i.e.*, broth containing either unheated or heated potato. If growth occurred in the second subculture, a third was made, the conditions of cultivation being kept constant throughout. These "aerobic" cultures of the various organisms will be called Subcultures 1, 2, and 3 respectively. By referring to these numerals one can determine by how many subcultures, under aerobic conditions, the organisms were removed from the original anaerobic potato broth cultures.

The final observations on growth were made after the tubes had remained in the incubator at 37°C. for 48 hours. Purity of the cultures was checked, in every instance, by examination of films stained by the Gram method.

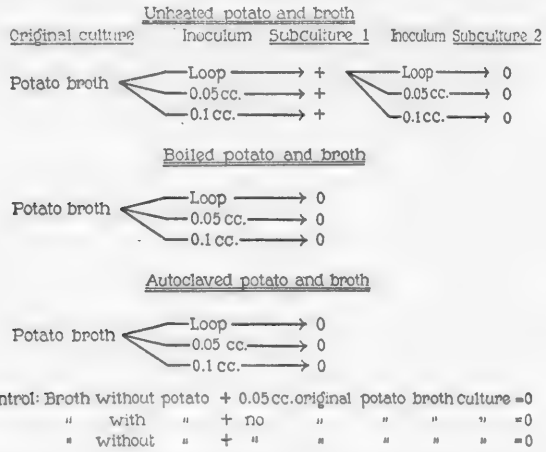
Results.—Bacterium pneumosintes (*Text-Fig. 1*): When a 2 mm. loopful of an 8 hour anaerobic potato broth culture of *Bacterium pneumosintes* was seeded into broth containing unheated plant tissue, under aerobic conditions, growth resulted. The culture showed marked clouding after 40 hours at 37°C. However, when as much as 0.1 cc. of the anaerobic culture was seeded into broth containing boiled or autoclaved potato, growth failed to occur.

When transfers were made from the aerobic Subculture 1 to Subculture 2 no growth occurred.

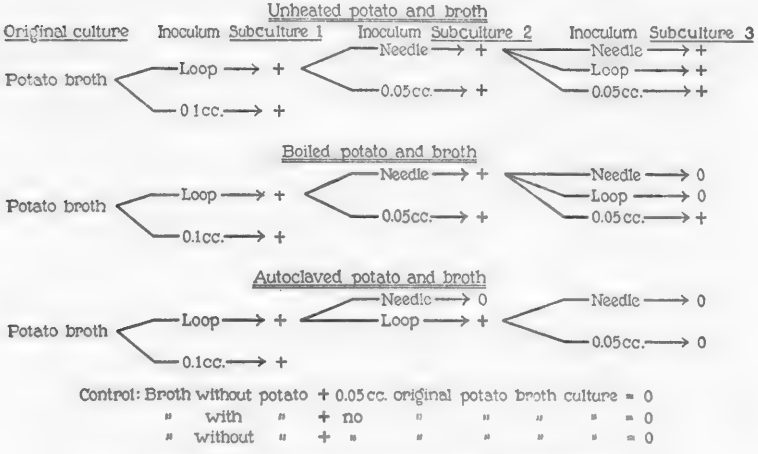
B. histolyticus (*Text-Fig. 2*): When *B. histolyticus* was transferred from an anaerobic potato broth culture into the three types of media, *i.e.* broth and unheated potato, broth and boiled potato, and broth and autoclaved potato, under aerobic conditions, growth occurred. When, in turn, organisms of Subculture 1

[2] The strain of *Bacterium pneumosintes* was furnished by Dr. P. K. Olitsky and Dr. F. L. Gates and had been grown in kidney tissue-ascitic fluid medium through many subcultures. For the other organisms we are indebted to Dr. Morton C. Kahn. Each strain was derived from a single cell culture and had been grown in cooked meat media for some time.

were transferred to a medium of the same type (Subculture 2), growth occurred in all cultures except in one of those containing autoclaved potato. The strain which


Unheated potato and broth
Original culture
Inoculum
Subculture 1
Inoculum
Subculture 2
Potato broth
Loop → +
0.05 cc. → +
0.1 cc. → +
Loop → 0
0.05 cc. → 0
0.1 cc. → 0
Boiled potato and broth
Potato broth
Loop → 0
0.05 cc. → 0
0.1 cc. → 0
Autoclaved potato and broth
Potato broth
Loop → 0
0.05 cc. → 0
0.1 cc. → 0
Control: Broth without potato + 0.05 cc. original potato broth culture = 0
" with " + no " " " " " = 0
" without " + " " " " " " = 0

TEXT-FIG. 1. Aerobic cultivation of *Bacterium pneumosintes*.

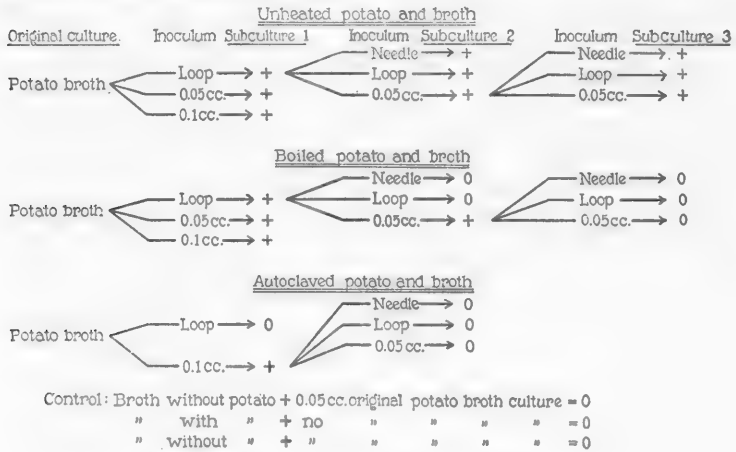


TEXT-FIG. 2. Aerobic cultivation of *B. histolyticus*.

had survived two transfers in broth containing autoclaved potato failed to grow in the third subculture even though a relatively large inoculum was used (0.05 cc.).

In the boiled potato medium growth did not occur in the third subculture when the inoculum was less than 0.05 cc., whereas the organisms carried over on the tip of a platinum needle sufficed to produce abundant growth in broth containing unheated potato.

B. chauvæi (*Text-Fig. 3*): The transfers of *B. chauvæi* in unheated potato broth media, under aerobic conditions, grew in the three subcultures. The strain seeded into boiled potato broth grew in all tubes of Subculture 1. In Subculture 2, however, growth could be initiated only when a relatively large inoculum was used (0.05 cc.), and no growth resulted in Subculture 3. When autoclaved potato was employed in the medium growth did not extend beyond Subculture 1, and there only when large inocula were used.



TEXT-FIG. 3. Aerobic cultivation of *B. chauvæi*.

B. aerofœtidis: Subcultures of this organism grew through three transfers, under aerobic conditions, in broth containing unheated and autoclaved plant tissue. In autoclaved potato broth growth was slow, not abundant, and occurred only in the tube which received the largest inoculum (0.05 cc.), whereas it was quite rapid (present after 15 hours at 37°C.) and abundant in broth containing unheated plant tissue, even when the latter had received the smallest inoculum. Growth of *B. aerofœtidis* did not continue beyond the first subculture in the medium composed of broth and boiled potato.

B. œdematiens: As in the case of the preceding organism, this anaerobe grew readily, under aerobic conditions, through three subcultures in broth containing unheated and autoclaved vegetable tissue. *B. œdematiens* like *B. aerofœtidis* grew in the autoclaved potato medium, but as in the latter instance the growth under these conditions was not so rapid nor so abundant as in the medium con-

taining unheated plant tissue. Boiled potato broth supported growth through two transfers only. An inoculum as large as 0.05 cc. failed to induce growth in Subculture 3.

To summarize, it was found that under the conditions of the experiment, the five strains of anaerobic organisms tested grew under aerobic conditions in broth containing pieces of unheated potato. In the case of *Bacterium pneumosintes*, growth did not continue beyond the first subculture. *Bacillus histolyticus*, *Bacillus chauvœi*, *Bacillus aerofœtidis*, and *Bacillus œdematiens* continued to multiply through three successive subcultures under aerobic conditions in the presence of unheated plant tissue.

Attempts to cultivate these organisms aerobically were not so successful when heated instead of unheated potato was used in the medium. *Bacterium pneumosintes* failed to grow in broth containing boiled or autoclaved potato. *Bacillus histolyticus* grew through three subcultures in boiled potato broth, but a larger inoculum was required to initiate growth in the third transfer. Likewise, in the medium containing autoclaved potato growth of this organism occurred aerobically in the second transfer only when a relatively larger seeding was added, and no growth took place in the third subculture even when the maximal inoculum was used. *Bacillus chauvœi* grew aerobically in the medium containing boiled potato through two transfers only and in the second subculture growth appeared only in the medium receiving the heavier seeding. When autoclaved potato was used this organism could not be induced to grow aerobically beyond the first subculture. Cultures of *Bacillus aerofœtidis* and *Bacillus œdematiens* were successfully carried through three aerobic transfers in broth containing autoclaved potato; growth under these conditions, however, was not so prompt and abundant as in the medium in which fresh unheated vegetable was used. In broth containing boiled potato both of these organisms grew less readily than they did in the same medium containing autoclaved potato. Cultures of *Bacillus aerofœtidis* and *Bacillus œdematiens* produced gas in broth containing either heated or unheated potato.

These results indicate that fresh, unheated plant tissue (potato) possesses to a striking degree the property of supporting the aerobic growth of bacteria ordinarily considered fastidious in their anaerobic

requirements. The fact that under aerobic conditions heated plant tissue exerts a less favorable action on the growth of anaerobic bacteria than does the unheated tissue, suggests that the superior growth value of fresh raw vegetable is associated with some heat-labile quality, possibly of the nature of an enzyme or group of enzymes. The loss of the peroxidase reaction in heated vegetable tissue is evidence of the susceptibility of the vegetable oxidases to the injurious action of heat. When potato is exposed to boiling water or to steam under pressure as in the present experiments, the oxidases of the vegetable tissue are destroyed. The relative effectiveness of unheated and heated potato in supporting the aerobic growth of anaerobic bacteria so closely parallels the presence or absence of these oxidases in the tissue as to suggest that the growth differences observed are related to the activity of these oxidizing enzymes. In the absence of these active enzymes, heated vegetable tissue supports only a limited and irregular development of these air-sensitive bacteria. The heated vegetable may function owing to the presence in it of certain stable tissue constituents which act as catalytic agents in reducing oxygen tension. Moreover, the interstices of the tissue afford opportunity for nidus formation which facilitates survival and multiplication of the microorganisms. In addition the bacteria themselves, when chance thus favors their increase, contribute to further growth by autocatalysis. Whatever share these special factors may have in the mechanism of growth, they are, presumably operative regardless of whether the tissue is heated or unheated. There is, however, an additional advantage in the use of the fresh raw vegetable, and experimental evidence indicates that this advantage is associated with the presence of the active oxidases of the unheated tissues. The possible relation of the oxidase system of plant tissue to bacterial growth will be discussed later in this paper. It suffices here to point out that such a relationship may exist and the oxidizing and reducing enzymes of unheated potato may function actively in creating cultural conditions suitable for the aerobic growth of organisms otherwise incapable of development in the presence of air.

In 1890 Theobald Smith, describing for the first time the influence of sterile animal tissue on the growth of anaerobic bacteria, suggested that the term anaerobiosis in bacteriology does not imply the impor-

tance of the absence of air so much as it infers the lack of knowledge of the particular cultural conditions requisite for the aerobic growth of certain types of bacteria.

DISCUSSION.

The growth-stimulating effect of plant tissue on bacteria of such widely diverse groups as those of the hemophilic bacilli, Gram-positive cocci, and anaerobic organisms serves to emphasize the need of a fuller knowledge concerning the physiology of the bacterial cell.

The studies presented in the present series of papers were undertaken with the hope that an analysis of the factors concerned in the mechanism of growth stimulation might lead to a better understanding of the principles involved in the action of these accessory substances in complementing the cultural requirements of bacteria. The fact that the addition of sterile unheated plant tissue to an otherwise deficient medium suffices to provide conditions suitable for cell multiplication, indicates that in bacterial as in animal growth there are substances which, apart from their food value, exert a marked influence upon the metabolism of the organism.

In order to correlate the facts presented in the present study with the observations recorded in the preceding papers it seems desirable to review briefly the work as a whole. Previous study of the growth requirements of *Bacillus influenzæ* has shown that the properties of blood upon which growth of this so called hemoglobinophilic organism depends, are related to two separable and distinct substances. The combined action of both of these substances is essential, each being ineffective separately. On the basis of their relative resistance to heat these two factors can be differentiated one from the other. The so called X factor, which is associated with the pigment fraction of blood, gives the peroxidase reaction, and is not destroyed by moist heat at 120°C.; while the second or so called V factor resists boiling for short periods, but is destroyed by exposure to the higher temperature. Because of this difference in susceptibility to heat, blood can be deprived of the more labile substance (V) by autoclaving and when so treated is no longer capable of supporting growth of *Bacillus influenzæ*. The X substance under these conditions, however, remains unaffected. This is proven by the fact that the addi-

tion of extract of yeast (V) can activate autoclaved blood (X) and render it again suitable for growth. It is interesting in this connection to note that, as Hopkins, Meyerhof, and others have shown, extracts of plant and animal tissue (yeast and muscle) are of physiological importance in cell respiration. This V substance which is capable of complementing the X factor of blood has been found in extracts of green vegetable and yeast cells, and from the nature of its action it may be regarded either as a coenzyme or as a vitamin-like substance. The X factor, on the other hand, is found in hemoglobin and in certain of its derivatives (hemin) and is always associated with the fraction giving the peroxidase reaction with benzidine. Moreover, the X substance is active in such minute amounts as to suggest that it functions as a biocatalyst.

Further study has confirmed the observation that the particular system represented by the combination of the X and V factors in blood has a complete analogue in a similar system of plant tissue. Unheated vegetable, for instance potato, possesses substances having the characteristics of both the X and V factors of blood. The peroxidase and catalase of plant tissue are related in function at least to the catalytic properties of the X factor in blood; while the so called V substance which serves as activator, both in blood and plant tissue, may represent a coferment adapted to a particular oxidase system. The mechanism of the action of plant tissue in stimulating growth of the hemophilic bacteria is not wholly clear. Analysis of the factors concerned, and the application of this knowledge to the growth of other bacteria, suggest that these factors may be related to the physiological processes of cell respiration.

It is of interest to correlate these facts with the effect of unheated vegetable tissue upon the growth of pneumococcus. It is, of course, well recognized that if a fluid medium be inoculated with an insufficient number of pneumococci little or no growth will occur, although the culture fluid may be optimal for growth if larger numbers of the organisms are introduced. However, if to this medium a piece of unheated potato is added, the minimal inoculum amply suffices to initiate prompt and abundant growth. Furthermore, the presence of plant tissue in the bouillon effects a remarkable alteration in the growth curve of pneumococcus. The period of lag, as pointed out

in the preceding paper (2), is eliminated, the maximum rate of cell division is promptly initiated, and growth reaches its full development in the first 8 hours of incubation. Moreover, sensitive as pneumococcus is to the initial reaction of the medium, the zone of hydrogen ion concentration within which growth can be initiated is considerably extended both on the acid and alkaline side in broth containing unheated vegetable tissue (potato). While the same mechanism which operates to facilitate growth of the hemophilic bacilli in plant tissue medium may be involved in the growth acceleration of pneumococci, in this latter instance another fact has been observed which may be of significance in explaining the action of these accessory factors. Whenever pneumococci are grown in bouillon to which there is free access of air, hydrogen peroxide is formed in readily demonstrable amounts (12, 13). This peroxide is toxic and accumulates in the culture fluid in concentrations which are bactericidal. On the other hand, under identical conditions of oxygen exposure, hydrogen peroxide is not demonstrable in cultures of pneumococci containing unheated plant tissue (potato). The absence of peroxide under these conditions is presumably due to the action of the catalase and peroxidase of the potato which possess the property of decomposing this substance. The presence of these enzymes, therefore, prevents the accumulation of hydrogen peroxide in the medium. The peroxidase in plant tissue is capable of acting upon the peroxide with the liberation of active oxygen which in turn can bring about further oxidative reactions. The results of studies on the formation of peroxide by pneumococcus and on the growth-inhibiting action of this substance on bacteria is described in another paper (13); it suffices here to point out that in the mechanism of growth acceleration of bacteria, plant tissue may exercise the dual function of providing accessory growth factors, and of destroying deleterious growth products—peroxides.

Finally, as demonstrated in the present paper, plant tissue makes possible the aerobic cultivation of anaerobic bacteria. The present study on the cultivation of anaerobes under these conditions was completed without knowledge of previous work on the use of plant tissue in the aerobic cultivation of anaerobic bacteria. Subsequent search of the literature revealed the interesting fact that 18 years ago

Italian bacteriologists of the Sienna school found that anaerobic bacilli of the obligate type grow aerobically in bouillon containing plant tissue. This phenomenon was attributed at that time to the action of reducing substances in the vegetable tissue.

The mechanism of aerobic growth of anaerobic bacteria in media containing pieces of unheated vegetable may, as already pointed out, be related to the oxidation-reduction system present in the plant tissue. Recent studies on the formation of peroxide by pneumococci on exposure to air and of the absence of this product when the organisms are grown anaerobically suggested the following assumption which finds some justification in the facts presented. Although experimental proof is not as yet available, it seems not unlikely that anaerobic organisms fail to grow in the presence of air not because atmospheric oxygen as such is a direct poison to the cell, but because of toxic peroxides which are produced whenever the oxygen of the air combines with autoxidizable substances of the bacterial cell. This process of autoxidation gives rise finally to the formation of hydrogen peroxide, which is known to be poisonous to living protoplasm. Since these peroxides are formed only by the action of molecular oxygen on some autoxidizable substance of the cell, the absence of air precludes their formation. Under anaerobic conditions, therefore, bacteria are not exposed to the injurious action of these products of autoxidation. If this assumption is correct, then the aerobic growth of obligate anaerobes in the presence of plant tissue finds partial explanation at least in the fact that the peroxide formed by autoxidation is rapidly broken up by the catalase and peroxidases of the plant tissue. Under these cultural conditions the sensitive cell is protected almost as effectively as if it were growing under anaerobic conditions. Not only is the catalase of the plant tissue capable of protecting the bacterial cell in this manner from the readily diffusible peroxide, but the peroxidase in splitting this substance liberates active oxygen which in turn is capable of bringing about oxidation of substances not readily oxidized by molecular oxygen. The peroxidase may further assist then in facilitating the transport of oxygen to other substances, and possibly to the bacteria themselves.

The evidence in favor of the hypothesis of peroxide formation during the processes of bacterial autoxidation is strong. It is not to

be inferred, however, that hydrogen peroxide is the only active agent resulting from these oxidation reactions. Previous studies have shown that hydrogen peroxide is demonstrable only in cultures in which the bacteria are devoid of catalase or in which the medium contains no efficient catalyst. If this compound were the only toxic peroxide formed during autoxidation, then the assumption of its deleterious action upon anaerobic bacteria when exposed to oxygen could only find support in the case of those organisms which possess no catalase. But it is generally recognized that a great variety of unstable peroxides may be formed as intermediary products in autoxidation. And so far as known, these other organic peroxides are not affected by catalase. It is possible that bacteria which are peculiarly air-sensitive contain autoxidizable substances which are markedly reactive with molecular oxygen and that as a result of this union there are formed toxic peroxides of an order unaffected by bacterial catalase.

The protective action of the vegetable oxidases under these circumstances can be explained by assuming that some efficient enzyme of this system, as peroxidase, brings about the decomposition of these inhibitory products. Bacteria of this type can grow, therefore, only when oxygen is excluded; that is, under conditions which render peroxide formation impossible, or when in the presence of air some mechanism is provided which inhibits the formation of these substances or renders them inert once they have been formed.

The views expressed in this paper are stated with full realization of their inadequateness to explain the complex nature of the phenomena of oxidation in the living bacterial cell; they are advanced, however, with the hope that they may serve to stimulate further work on this important phase of the physiology of bacteria.

In the present state of knowledge it seems reasonable to assume that the growth-promoting properties of fresh plant tissue may be referable to the action of the oxidation-reduction system of the tissue as well as to the presence of other growth accessory substances which together provide cultural conditions more suitable for normal functioning of bacterial cells than are ordinarily furnished in bacteriological media.

Although the views expressed in this paper concerning the mechanism of anaerobiosis were arrived at independently, they are in striking accord with and indeed lend support to the studies recently published by McLeod and Gordon (14) and by Callow (15) on the oxygen intolerance of anaerobic bacteria. Callow made comparative estimations of nine anaerobes and of twelve aerobes and found that none of the anaerobic organisms contained catalase, while all of the aerobic bacteria, with the exception of the streptococci, showed the presence of this enzyme. In no instance, however, was she able to obtain conclusive evidence that anaerobes are able to grow aerobically in broth containing catalase derived from bacteria, fat, and yeast. Moreover, repeated attempts to detect hydrogen peroxide in cultures of anaerobes which had been subsequently exposed to oxygen were negative. McLeod and Gordon, following their original investigations on the catalase production and the formation of hydrogen peroxide by bacteria, have studied the influence of liver catalase on the development of anaerobes in the presence of oxygen. Although all attempts to cultivate anaerobes aerobically on plates heavily charged with liver catalase failed, these authors do not consider that this invalidates their theory that "anaerobes cannot tolerate more than very slight concentrations of oxygen because they produce H_2O_2 as soon as oxygen is available and being very sensitive to this substance they die." The failure of the English investigators to effect aerobic growth of anaerobes by addition of catalase when contrasted with successful results obtained by the use of fresh plant tissue, suggests that possibly the complete oxidation-reduction system in the latter is able to decompose toxic peroxides of an order unaffected by catalase alone.

CONCLUSIONS.

1. Certain anaerobic bacteria grow under aerobic conditions in plain broth in the presence of sterile unheated plant tissue.

2. Aerobic growth of anaerobic bacteria under the above conditions may be related to the presence of growth-accessory substances, and to the action of oxidizing-reducing systems of plant tissue in the destruction of toxic peroxides of bacterial origin.

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PROTEINURIA IN NEW-BORN CALVES FOLLOWING THE FEEDING OF COLOSTRUM.

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During the past 5 years about a hundred calves have been autopsied at the Department during the 1st week of life, usually within the 4 first days. Many of these calves were normal, *i.e.* active, alert, and consuming the usual amount of milk, and their flesh destined for use in the preparation of culture media. Some were manifestly ill, the most frequent symptoms being diarrhea. They were killed by being first stunned with a blow on the head and then bled by section of the vessels of the neck. In many cases urine taken from the bladder within an hour after they had been killed contained coagulable protein varying in amount from merest traces to enough to solidify completely the urine when acidified with a few drops of acetic acid and brought to the boiling point.

This proteinuria was absent in calves over 3 days old, if we except a very fine clouding often present after the heat test. It was for a time regarded as a premonitory sign of "scours," or the early diarrhea or cholera of calves, until one animal carefully watched lived through this period without manifesting any signs of illness. The protein was present on the 1st day of life but disappeared on the 3rd day.

The proper interpretation of this phenomenon was delayed by difficulties in the collection of urine. Even a crate to hold the calf so constructed as to save the urine failed to serve the purpose until it was found that a slight irritation of the external genitals brought a flow of urine in nearly all cases.

Another hypothesis that the urine contains protein at birth was negatived by the absence of all but traces in urine taken from the

bladder of fetuses near full term and of new-born calves that had not yet taken any food.

The distended fourth stomachs frequently encountered at the autopsies, filled with colostral milk, and the presence of coagulable protein in the contents of the ileum suggested the hypothesis that the protein in the urine might be associated with the colostrum. This view was strengthened by the rapid passage of antibodies and globulins from the digestive tract into the blood during the early days of life. To test this hypothesis, calves were taken immediately after birth, some fed with definite amounts of colostrum, others during the 1st day with milk and then colostrum. In several cases the feeding of colostrum was repeated after an interval of several days. In two cases cow serum took the place of colostrum. The latter was drawn from the dam's udder and refrigerated until needed. It was then warmed to 37°C. and fed from bottles provided with nipples. This method of handling had no injurious effect on the agglutinins as the individual records show. The ordinary milk was taken from cows well along in lactation. It was fed either immediately after the milking or else stored in the refrigerator for some hours and warmed before use. It also was fed from bottles in the early days of life. It was not pasteurized at any time.

The calves were fed three times daily, at 5 a.m., noon, and 5 p.m. In some instances a slight shift was necessary.

For the determination of agglutinins and blood proteins, each calf was bled two or three times during the early days of life. The sample amounting to from 25 to 50 cc. was taken from the jugular vein through a needle thrust through the skin into the vein. Since all calves were treated nearly alike in this respect, a statement of the collection of blood is omitted from the protocols.

Samples of urine were collected three times daily or oftener when obtainable. The amount of coagulable protein was determined first by acidifying about 10 cc. with 1 or 2 drops of acetic acid and bringing the fluid to the boiling point in a water bath. The column of precipitate was measured next day. The Esbach tube was used on all samples and the per cent of protein given below refers to this tube. In some samples the Esbach precipitate did not settle out completely. In others it was fluffy or curdy. These variations may be related to the varying amounts of globulins and albumin present. In the individual histories data concerning the reaction of the urine to litmus, the specific gravity, etc., are omitted.

Calves Fed Soon after Birth with Colostrum Varying in Amount from Case to Case.

500 Cc. of Colostrum. No. 830.—Holstein male calf, weighing 85 pounds, born Jan. 8, 10.20 a.m. Posterior presentation. Delivered by traction. 11.10 a.m. 500 cc. of dam's colostrum fed with the bottle. After this first limited meal of colostrum only milk is fed. 4.50 p.m. Urine contains about 0.06 per cent protein. Fed 3 pounds of milk. 9 p.m. Urine as before.

Jan. 9. Samples of urine collected at 5.40 and 9 a.m. contain 0.01 to 0.02 per cent protein. Diarrhea sets in and lasts less than 24 hours.

Jan. 12. Urine collected Jan. 10, 11, and today negative.

Jan. 23. The calf has been normal since last date, taking gradually increasing quantities of milk to a maximum of 15 pounds yesterday. Killed today and organs found normal.

Nearly 2 Liters of Colostrum. No. 660.—Holstein female calf, weighing 88 pounds, born Jan. 29, 11 a.m. 11.40 a.m. Fed 1,145 cc. of colostrum. 5.50 p.m. Fed 700 cc. of colostrum.

Jan. 30. 5.30 a.m. Milk feeding begun. Protein in urine at 8.20 a.m. was 0.2 per cent, at 12.30 p.m., 0.1 per cent, and at 2.30 p.m., 0.05 per cent.

Jan. 31. 5.40 a.m. 0.2 per cent protein in urine. Diarrhea set in last night and continues during the day.

Feb. 2. Calf died between 2 and 4.30 a.m. Had been growing weaker with the continuing liquid discharges. Organs found sterile in cultures made at autopsy. Urine taken from the bladder contained only a faint trace of protein.

Over 2 Liters of Colostrum. No. 1030.—Holstein female calf, weighing 68½ pounds, born Mar. 16, at 11.10 a.m. Taken soon after to a Department unit, dried by gentle rubbing with towels. 12.15 p.m. Fed from a bottle 1,200 cc. of dam's colostrum after it had been warmed to about 37°C. 3.30 p.m. Calf appears weak and able to stand up only for short periods. 4.50 p.m. Fed 1,000 cc. of colostrum drawn from the dam about 3 hours previous and warmed to 37°C.

Mar. 17. Feeding milk three times daily began at 5 a.m. Calf brighter and more active than yesterday. 8.15 a.m. Urine contains over 1 per cent protein. 5.15 p.m. Urine contains protein. The Esbach precipitate does not subside.

Mar. 18. Three samples of urine collected today show moderate clouding after the heat test.

Mar. 19 and 20. Urine negative.

Mar. 24. Animal has been normally active since the former date and taken its food regularly, but the temperature has been about 1°C. above normal. Began to have liquid stools this morning.

Mar. 27. The stools have become firmer today. Calf takes its food regularly.

Mar. 28. Used in another experiment.

Nearly 4 Liters of Colostrum. No. 708.—Holstein male calf, weighing 104 pounds, born Feb. 8, 10.30 a.m. Not permitted to suckle. Colostrum taken

from the udder and fed from a bottle. 11.20 a.m. 1,135 cc. of colostrum fed. 4 p.m. 0.1 per cent protein in the urine. 5.15 p.m. 1,000 cc. of colostrum fed.

Feb. 9. 8.10 a.m. 1,000 cc. of colostrum fed. 8.20 a.m. Urine contains 0.75 per cent protein. 12.30 p.m. 700 cc. of colostrum fed. 1.35 p.m. 0.6 per cent protein in urine. 5 p.m. Ordinary milk feeding begun. 5.25 p.m. 0.1 per cent protein in urine.

Feb. 10. Samples of urine collected at 6 and 11 a.m. contain approximately 0.05 and 0.01 per cent protein. Diarrhea noticed towards noon.

Feb. 11. Diarrhea ceases during the day. Two samples of urine collected at 6 and 11 a.m. negative.

Feb. 28. Calf has remained well. Killed today. Autopsy negative.

The antibodies towards *B. abortus* were low in the colostrum (1:40). None were in the calf's blood at birth but in the morning of the 2nd day they were present in a concentration of 1:10.

5 Liters of Colostrum. No. 1026.—Grade Holstein and Ayrshire male calf, weighing 96 pounds, born Mar. 8, 7.30 a.m. 9.10 a.m. Fed 1,225 cc. of dam's colostrum. 3 p.m. Urine contains 0.5 per cent protein. 3.20 p.m. One liter of colostrum fed. 9.30 p.m. One liter of colostrum fed.

Mar. 9. 5 a.m. One liter of colostrum fed. 11.30 a.m. One liter of colostrum fed. 2.30 p.m. Urine had to be diluted with an equal volume of normal salt solution owing to concentration of protein. Content about 1.6 per cent. 5 p.m. Milk feeding begun.

Mar. 10. 8 a.m. Urine contains about 0.05 per cent protein. 12 noon. Urine contains about 0.04 per cent protein.

Mar. 11. 5.40 a.m. Only a trace of protein in urine.

Mar. 12. 5.40 a.m. Very faint clouding after the heat test.

Mar. 13. 6.10 a.m. Urine negative.

Mar. 16. Some liquid stools today.

Mar. 17. Normal. Used in another experiment.

In this group which was fed soon after birth with colostrum withdrawn from the dam the amount of protein in the urine in general ran parallel with the amount of colostrum taken. The protein disappeared promptly after the feeding of ordinary milk had begun. One calf receiving about a liter died when between 3 and 4 days old, another receiving only 500 cc. survived.

Delayed Feeding of Colostrum.

In the two following cases, the feeding of colostrum was delayed to determine the condition of the urine.

No. 1031.—Holstein female calf, weighing 70 pounds, born Mar. 19, 11 a.m. Taken from the dam at once and placed in a Department unit, to be fed colostrum when about 20 hours old. The colostrum was taken from the dam's udder during the day and refrigerated. 12.05 p.m. About 500 cc. of milk fed to calf. 5.10 p.m. Repeated the same dose of milk. Three samples of urine collected during the day free from protein.

Mar. 20. 7 a.m. A sample of urine free from protein. Calf fed 1,500 cc. of colostrum. 12.20 p.m. Another meal of 1,500 cc. of colostrum given. 5 p.m. A third dose of 500 cc. of colostrum fed. A sample of urine contains 0.3 per cent protein.

Mar. 21. Diarrhea sets in and food somewhat restricted. About 1½ pounds of milk fed at 9 a.m. Heavy, flocculent precipitate in urine after boiling. 5 p.m. Feces watery. About 1½ pounds of milk fed.

A sample of urine collected Mar. 22 and one collected Mar. 23 show only a faint cloudiness after acidifying and boiling.

Mar 24. Diarrhea has continued since last date. Today feces more consistent. Milk continues to be fed three times a day in small quantities (1½ pounds).

Mar. 31. Feces more or less fluid since last date, but calf appears active and fairly strong. Today feces nearly normal.

Apr. 9. Calf has been normal since last date. Killed today. Autopsy negative.

No. 930.—Holstein male calf, weighing 77 pounds, born Apr. 13, 1.50 p.m. 3.30 p.m. Calf takes 450 cc. of milk from bottle. 9.30 p.m. Urine free from protein. 450 cc. of milk fed.

Apr. 14. 5.40 a.m. 450 cc. of milk fed. Urine free from protein. 12.30 p.m. Urine free from protein. 1,500 cc. of colostrum fed (22 hours after birth). 8 p.m. 1,000 cc. of colostrum fed. Urine contains 0.1 per cent protein. Diarrhea sets in.

Apr. 15. 5 a.m. 1,000 cc. of colostrum fed. 5.45 a.m. Urine diluted to one-fifth with normal salt solution yields 0.8 per cent protein. 11.25 a.m. 1,000 cc. of colostrum fed. Urine diluted to one-sixth yields 0.5 per cent protein. 5 p.m. Milk feeding begun.

Apr. 16. 8.45 a.m. Diarrhea continues. Undiluted urine contains 0.22 per cent protein. 11.40 a.m. Urine contains only 0.05 per cent protein.

Apr. 17. 5.35 a.m. A slight flocculent precipitate in urine after the heat test.

Apr. 18. 5.30 a.m. Only a trace of protein.

Apr. 19. 5.30 a.m. Urine free from protein.

Apr. 22. Diarrhea has continued interruptedly. Calf has, however, taken milk regularly and has appeared fairly active and strong. Today feces nearly normal.

May 1. Calf normal since last date.

In one case (No. 1031) colostrum was given when the calf was 20 hours old, in the other (No. 930) when it was 22 hours old. In both diarrhea appeared and continued for some days. The urine was free from all but faint traces of protein until after colostrum had been fed. Subsequent events were then like those observed in the preceding group.

Repeated Feeding of Colostrum.

In the following two cases the calf soon after birth was allowed to suckle the dam but once. After a period of ordinary milk feeding colostrum was again fed.

No. 927.—Holstein female calf, weighing 78 pounds, born May 1, 12.30 p.m. Within ½ hour it was held up and allowed to suckle the dam for about 25 minutes, more or less intermittently. 2 p.m. Sample of urine contains 0.05 per cent protein. 4.55 p.m. Calf took only 6 ounces of milk. 5 p.m. Urine contains 0.1 per cent protein.

May 2. Takes 1½ pounds of milk at 5 and 9 a.m. and at 5 p.m. Urine at 5.35 a.m. diluted to one-fourth contained 0.2 per cent protein; at noon a sample contained 0.18 per cent, and at 5 p.m. only a trace.

May 3. Calf fed as yesterday. No diarrhea. Two samples of urine contain only a trace of protein.

May 4. 8.45 a.m. Calf is fed 1,000 cc. of colostrum. The same amount fed at 2 and 8 p.m. Feces liquid at this hour. Five samples of urine collected during the day free from protein.

May 5. 5 a.m. 1,000 cc. of colostrum fed to calf. Diarrhea during the day. 5.30 a.m. Urine contains 0.08 per cent protein. 11.30 a.m. Milk feeding begun again.

May 9. Feces today nearly normal. More or less fluid since last date. Calf bright and active. One sample of urine contains a trace of protein.

May 10. Urine free from protein.

May 22. Calf has been normal since preceding date, taking 9 to 10 pounds of milk daily. Killed today. Autopsy negative.

No. 1059.—Holstein female calf, weighing 83 pounds, born May 16, 3.30 p.m. The calf suckled the dam from 4.10 to 4.55 p.m. at intervals.

May 17. Calf fed about 700 cc. of milk three times today. Urine at 5.30 a.m. contains enough coagulable protein to form a 12 per cent deposit in the test-tube. The sample of 11.30 a.m. had a deposit of 5 per cent. At 5 p.m., a sample contained only a trace.

May 18. Milk feeding continued today. Urine of 5.40 a.m. free from protein.

May 19. 5.30 a.m. Receives 700 cc. of milk. Urine of this hour free from protein. 12 noon. Colostrum from another cow obtained and about 700 cc.

fed. 4.50 p.m. Urine contains a mere trace of protein. 7.30 p.m. 900 cc. of colostrum fed. 10.55 p.m. Urine boiled contains a precipitate equivalent to one-fourth of the fluid column.

May 20. 5 a.m. 1,350 cc. of colostrum fed. 5.40 a.m. Urine contains 0.1 per cent protein. 11.10 a.m. Urine contains 0.15 per cent protein. 11.30 a.m. 1,350 cc. of colostrum fed. 4.45 p.m. Urine contains 0.28 per cent protein. 5 p.m. Milk feeding resumed.

May 21. Two samples of urine collected at 6 a.m. and 12 noon contain protein equivalent to about 5 per cent of the fluid column.

May 22. Only one sample of urine obtained (6 a.m.); contains about 0.05 per cent protein.

Samples collected May 23, 24, and at 5.45 a.m., May 25, yield only a very faint clouding after the heat test.

May 25. 4.45 p.m. Colostrum feeding resumed. Colostrum collected from a cow before parturition because of overdistension of the udder. Fluid rather thin. 1,500 cc. fed.

May 26. 5 a.m. Only a faint clouding of the urine following the heat test. 1,500 cc. of colostrum fed. 5 p.m. Urine as before. Milk feeding resumed.

May 29. Samples of urine collected daily once or twice since May 26 yielded only a very faint clouding after the heat test. Today colostrum feeding resumed. About 1,500 cc. from a fourth cow fed at noon and at 5 p.m.

May 30. 5 a.m. 1,500 cc. of colostrum fed. 11.30 a.m. Milk feeding resumed.

May 31. Urine collected May 29, late, and on May 30, and today contained only faint traces of protein.

June 11. Calf has been normal since preceding date, except for a swelling of both cheeks which was caused by ulceration of the mucous membrane induced by slightly tilted molars. The urine examined daily shows either a very faint clouding following the heat test or else remains clear.

June 12. Calf killed today. Organs normal with exception of ulcers referred to above.

In No. 927, colostrum was fed once immediately after birth and when the calf was 2 days and 20 hours old. Protein appeared after the first meal, the maximum observed being 0.18 per cent. After the second feeding, in only one sample of urine obtained the amount was 0.08 per cent.

In No. 1059, colostrum was fed once immediately after birth and repeated three times. After the first natural feeding, protein appeared in the urine as in earlier cases. After the second feeding, when the calf was nearly 3 days old, protein reappeared and continued 3 days. The third colostrum period began when the calf was 9

days old. The urine showed either a very faint clouding following the heat test or else remained clear. The same is true of the samples following the fourth period. The kidneys thus remained pervious in this case for 6 days after birth.

Cow Serum in Place of Colostrum.

In the two cases following, cow serum was fed about 3 hours after birth in one case, and in the other about 21 hours after birth.

No. 1034.—Holstein female calf, weighing 71 pounds, born Apr. 9, at 1.30 p.m. Separated from the dam at birth. 4.35 p.m. About 1,000 cc. of serum of a cow immunized to *B. abortus* fed to calf. 10.30 p.m. A meal of 300 cc. of cow serum and 700 cc. of milk mixed, fed to calf.

Apr. 10. 5 a.m. 2 pounds of ordinary milk fed.

Apr. 11. Calf has been normal until this morning when diarrhea set in. Food taken regularly however.

Apr. 12. Some blood noticed in the soft feces. Calf active and hungry for its food consisting of 2 pounds of milk three times daily.

Apr. 19. Calf has been passing fluid feces now and then to date. Today condition normal. Calf takes about 2½ pounds of milk for a meal.

Apr. 23. Has been growing stronger and quite active. Fed about 9 pounds of milk per day. Killed today. Autopsy negative.

The urine in this calf responded to the feeding of serum as follows: A sample before the first feeding became very faintly clouded when the heat test was used. 6 hours after the serum meal, the clouding was slightly increased. 10 hours after the first and 7 hours after the second serum meal, the heat test gave a heavy precipitate, equivalent to nearly one-fourth the fluid column. In the Esbach tube the fluid remained turbid and 0.12 per cent subsided. After this time, the urine showed only faint clouding when slightly acidified and boiled until April 13 when it remained clear after heating.

The serum fed contained not only agglutinins to *Bacillus abortus*, but also to a colon bacillus frequently found in calf septicemia. The blood of the calf before feeding contained neither. 6 hours after feeding the first meal of serum the blood titer of the calf towards *Bacillus abortus* and *Bacillus coli* was 1:320.

No. 859.—Holstein male calf, born Jan. 24, at 2.50 p.m. Very weak at birth, just able to stand up. 4 p.m. Fed 750 cc. of milk. The same dose given at 10 p.m.

Jan. 25. 11.30 a.m. A mixture of 500 cc. of milk and 500 cc. of serum from the same cow supplying serum for No. 1034, fed. Diarrhea begins. 3.50 p.m. The same quantity of milk and serum fed. 11 p.m. The same quantity of milk and serum fed.

Jan. 27. Calf has been taking its milk regularly since preceding date and has been fairly active. Today it is weaker, the stools are fluid with traces of blood. (The same condition noted yesterday.) Joints stiff.

Jan. 28. Has taken its milk regularly, but is weak and dull; head extended, eyes sunken. Respirations increased in number. Refuses its food towards evening. Respiration labored, joints tender.

Jan. 29. Calf died at 2.30 a.m. Refrigerated. At the autopsy there were noted edema of lungs, intense congestion of kidneys, and fibrinous exudate in joints. Cultures showed a *B. coli* septicemia.

The samples of urine collected two or three times a day did not at any time contain more than a mere trace of coagulable protein.

Following the feeding of serum, the agglutinins towards *B. abortus* rose in the blood of the calf from less than 1:5 to 1:10.

It is probable that No. 859 would have survived if the serum had been given soon after birth. The absorption of the delayed serum was probably very slight in view of the feeble response in the urine, the low agglutinins, and the *Bacillus coli* septicemia. The diarrhea in No. 1034 might have been prevented with a larger dose of serum.

Control Cases.

In the following case, the calf received only milk. The urine was negative and the calf died of *Bacillus coli* septicemia.

No. 1020.—Holstein female calf, weighing 91 pounds, born Jan. 18, between 9 and 10 a.m. This calf was not permitted to suckle but was fed ordinary milk. Diarrhea began towards noon. The calf showed signs of toxemia which disappeared with the diarrhea next day. Diarrhea reappeared Jan. 21, the calf grew rapidly weaker, and died at 10.30 p.m. The autopsy and cultures showed a *B. coli* septicemia. Only two samples of urine were obtained, both on the morning of Jan. 20, when the calf was 2 days old. In both only very faint clouding followed the heat test.

There may be mentioned here as controls three calves, two of which were with the dam for some hours and had suckled, and a third which being too weak was held up to the udder to feed. These were taken to the Department units and milk feeding begun. In samples of urine collected for several days, coagulable protein was present on the 2nd day of life in amounts from 0.2 to 0.3 per cent.

SUMMARY AND CONCLUSIONS.

The evidence presented indicates clearly that the feeding of colostrum is responsible for the proteinuria. Observations on many calves killed within the 3 first days have shown that this condition is nearly universal and that it disappears after the 3rd day, even when the calf is not quite normal. Urine of fetuses and unfed calves did not contain it. By feeding different amounts of colostrum, by withholding it for some hours after birth, and by giving it alternately with milk, the appearance of protein in the urine as to time and quantity was clearly a function of the colostrum fed. Serum replacing colostrum produced the same effect to a less pronounced degree.

The permeability of the kidneys as determined in a routine way on healthy calves killed within the 1st week of life ceases after the 3rd day. In the two cases fed repeatedly with colostrum, proteinuria was demonstrated up to 6 days in one case. The regularity of its disappearance in calves normally fed indicates that more experiments are necessary to prove a longer period of permeability. The continued excretion of protein in such animals may be due to a slight lesion of the kidneys.

There is but one reference to this subject which we have been able to find. Langstein and Neuberg[1] in a study of levulose and allantoin in the urine of new-born calves mention the presence of albumin in six cases without, however, making any comments as to its origin or referring it to the ingestion of colostrum. According to various observers,[2,3] albumin may be found in traces during the 4 first days in over 60 per cent of infants examined. Since the food has not been taken into consideration renewed observations are needed to determine if the relationship found in calves is also true for the human infant.

[1] Langstein, L., and Neuberg, C., *Biochem. Z.*, 1907, iv, 292.
[2] Gundobin, N., *Arch. Kinderheilk.*, 1907, xlvi, 267.
[3] Heller, F., *Z. Kinderheilk.*, 1913, vii, 303.

THE RELATION BETWEEN THE INGESTION OF COLOSTRUM OR BLOOD SERUM AND THE APPEARANCE OF GLOBULIN AND ALBUMIN IN THE BLOOD AND URINE OF THE NEW-BORN CALF.

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In connection with a problem relating to the ingestion of colostrum and the appearance of protein in the urine of calves, analyses have been made of the proteins in the blood of the calves studied and also a differential analysis of the proteins of the urines in at least one urine in each case. A preceding paper contains the observations made with regard to the details of feeding, the time of appearance and disappearance of the protein in the urine, and discussions of the cases (1).

Presentation of Data.—The blood serum was analyzed according to procedures which have been described (2). The urines were analyzed according to similar procedures. The accuracy of the method in the case of urine was tested by adding blood serum to the urine and analyzing the mixture; with the samples of urine used the distribution of the proteins agreed with the analysis of the blood alone. When small amounts of protein were present the urines were fractioned by adding the proper amount of solid sodium sulfate. It is possible that in such cases, when the concentration of urea was high, the procedure was only approximate. On the other hand, when the protein content was high enough so that but 0.5 cc. of urine to 15 cc. of salt solution could be used, the interference of other urine constituents was negligible.

Calves Fed Colostrum.—The blood and urine of sixteen cases were studied in which calves ingested colostrum by nursing the dam or from a bottle. In each case the appearance of the globulin fractions in the blood (3) and of the various protein fractions in the urine was demonstrated. Data from representative cases are included

in the accompanying tables. Analyses of the colostrum fed are also included. The data in Tables I to IV show that the quantity of blood globulin remains at a relatively high level after protein has disappeared from the urine. The gradual disappearance of absorbed

TABLE I.

*Calf 708. Fed Colostrum Soon after Birth.**

Calf born Feb. 8, 10.30 a.m. It was fed colostrum as follows: Feb. 8, 11.35 a.m., 1,135 cc., 5.15 p.m., 1,000 cc.; Feb. 9, 8.10 a.m., 1,000 cc., 12.30 p.m., 700 cc.

Urine.—A positive test for protein was obtained up to 11 a.m., on Feb. 10; the next sample, 6.10 a.m., Feb. 11, was negative.

Sample collected.	Total N.	Serum N.	Euglobulin N.	Pseudoglobulin N. I	Pseudoglobulin N. II	Total globulin N.	Albumin N.	Nonprotein N.
Blood.								
Feb. 8, 11.20 a.m.		0.699	0.009	0.057	0.113	0.179	0.488	0.032
Fed colostrum at 11.35 a.m., Feb. 8.								
Feb. 9, 7.50 a.m.		0.855	0.137	0.179	0.136	0.452	0.391	0.012
" 12, 2.30 p.m.		0.770	0.125	0.108	0.101	0.334	0.412	0.024
" 28	0.894	0.839	0.085	0.129	0.093	0.307	0.508	0.024
Urine.								
Feb. 8, 4.00 p.m.	0.294		0.012	0.009	0.004	0.013	0.006	0.263
" 9, 8.20 a.m.	0.895		0.053	0.040	0.034	0.117	0.167	0.610
" 10, 6.15 "	0.764		0.046			0.064	0.076	0.624
Colostrum.								
	2.304	Casein. 1.024	0.388	0.364	0.189		0.283	0.048

*In this and the following tables the results are expressed as grams of nitrogen per 100 cc.

protein from the blood has been previously demonstrated (4). The data in Table III are presented to show that the permeability of the kidney as well as of the alimentary tract persists for some time. In this case the feeding of colostrum was delayed for 22 hours after birth during which time milk was fed. Following the ingestion of colostrum there was an accumulation of protein in the blood and the various protein fractions appeared in the urine.

Two facts are brought out in Table IV: (*a*) After a calf has received colostrum and protein has appeared in the blood and in the urine and has disappeared from the urine, a second ingestion of colostrum will be followed by an increase in protein in the blood and the reappearance of protein in the urine. (*b*) The time during which absorption from the intestinal tract can take place may be as long as 69 hours.

TABLE II.

Calf 1026. Fed Colostrum Soon after Birth.

Calf born Mar. 8, 7.30 a.m. Fed colostrum as follows: Mar. 8, 9.10 a.m., 1,225 cc., 3.20 p.m., 1,000 cc., 9.30 p.m., 1,000 cc.; Mar. 9, 5 a.m., 1,000 cc., 11.30 a.m., 1,000 cc.

Urine.—Protein positive in the urine at 12 noon, Mar. 10, and the next sample at 5.40 a.m., Mar. 11, negative (trace).

Sample collected.	Total N.	Serum N.	Euglobulin N.	Pseudoglobulin N.		Total globulin N.	Albumin N.	Nonprotein N.
				I	II			
Blood.								
Mar. 8, 9.00 a.m.		0.684	0.000	0.047	0.141	0.188	0.468	0.028
Fed colostrum immediately after bleeding.								
Mar. 8, 3.20 p.m.		0.833	0.115	0.173	0.105	0.393	0.316	0.024
" 10, 10.00 a.m.		1.005	0.160	0.232	0.145	0.537	0.396	0.072
Urine.								
Mar. 8, 3.00 p.m.	0.383		0.044	0.011	0.009	0.062	0.020	0.301
" 9, 2.30 "	1.535		0.101	0.059	0.086	0.256	0.132	1.147
" 10, 8.00 a.m.	0.486		0.012	0.012	0.274	0.298	0.030	1.516
Colostrum.								
	2.455	Casein. 0.783	0.786	0.094	0.009	0.889	0.637	0.032

Calves Fed Cow Serum.—In two cases blood serum of the cow was fed to calves instead of colostrum. In the first case 500 cc. of blood serum was fed to a calf 21 hours after birth. There was no appreciable rise in the proteins of the blood nor did protein appear in the urine in more than traces. There may have been greater difficulty in absorbing protein at 21 hours than at birth, but the results

obtained agree with unpublished data in which the ingestion of 500 cc. of blood serum soon after birth was not sufficient to cause a measurable increase in the blood proteins of calves. The second case in which blood serum was fed was successful in indicating the

TABLE III.

Calf 930. First Fed Colostrum 22 Hours after Birth.

Calf was born Apr. 13, at 1.50 p.m. It was fed milk until 12 noon, Apr. 14, when it was given 1,500 cc. of colostrum. Colostrum (1,000 cc.) was again fed at 8 p.m., and on Apr. 15 at 5 a.m. and at 11.25 a.m.

Urine.—Protein in the urine was negative until 8.20 p.m. on Apr. 14, when the tests became positive and remained so until 5.35 a.m. on Apr. 17, at which time there was a slight trace of protein.

Sample collected.	Total N.	Serum N.	Euglobulin N.	Pseudoglobulin N.		Total globulin N.	Albumin N.	Nonprotein N.
				I	II			
Blood.								
Apr. 13, 3.30 p.m.		0.723	0.000	0.057	0.166	0.213	0.451	0.059
" 14, 11.45 a.m.		0.728	0.031	0.030	0.161	0.222	0.451	0.055
Fed colostrum 22 hrs. after birth.								
Apr. 14, 8.00 p.m.		0.884	0.030	0.161	0.140	0.331	0.498	0.055
" 15, 11.25 a.m.		0.981	0.225	0.097	0.149	0.471	0.458	0.052
" 16, 9.10 "		0.902	0.069	0.208	0.124	0.401	0.429	0.072
May 23		0.917	0.044	0.125	0.092	0.261	0.603	0.053
Urine.								
Apr. 15, 11.35 a.m.	2.085		0.000	0.098	0.212	0.300	0.310	1.475
Colostrum.								
		Casein.						
Apr. 14	3.238	1.309	1.007	0.327	0.217	1.551	0.281	0.097

appearance of protein in the blood and the excretion of protein in the urine. In this case larger amounts of blood were fed, 1,000 cc. and later 300 cc. The data are contained in Table V.

DISCUSSION.

The data presented support the inference that the protein present in the urine of new-born calves which have received colostrum is

derived from the colostrum as follows: (*a*) In every uncomplicated case in which protein has appeared in the urine of new-born calves following the ingestion of colostrum, euglobulin and pseudoglobulin I have appeared in the blood (4). (*b*) When colostrum has been with-

TABLE IV.

Calf 927. The Effect of a Second Ingestion of Colostrum after Protein Had Disappeared from the Urine, as the Result of a First Ingestion.

Calf was born May 1, at 12.30 p.m., and allowed to suckle the dam. Subsequently it was fed milk until protein disappeared from the urine, when it was again fed colostrum (69 hours old) as follows: May 4, 9.10 a.m., 1,000 cc., 2 p.m., 1,000 cc., 8. p.m., 1,000 cc.; May 5, 5 a.m., 1,000 cc.

Urine.—Protein was present in the urine from 2 p.m. on May 1, to 5 p.m. on May 2, when it was negative (trace). Following the second feeding of colostrum the urine became positive, May 5, 4.30 a.m., and was again negative (trace) on May 9.

Sample collected.	Total N.	Serum N.	Euglobulin N.	Pseudoglobulin N.		Total globulin N.	Albumin N.	Nonprotein N.
				I	II			
Suckled dam at birth.								
Blood.								
May 1, 4.55 p.m.....		0.743	0.025	0.030	0.140	0.199	0.489	0.055
" 4, 8.45 a.m.....		0.765	0.030	0.013	0.199	0.242	0.485	0.038
Fed colostrum at 9.10 a.m., 2 p.m., and 8 p.m. on May 4, and at 5 a.m. on May 5.								
May 5, 9.30 a.m.....		0.789	0.118	0.083	0.157	0.258	0.463	0.068
" 22.....		0.803	0.115	0.051	0.111	0.317	0.544	0.042
Urine.								
May 2, 5.35 a.m.....	1.011					0.148	0.091	0.772
" 5, 5.30 "	0.961					0.434	0.037	0.490
Colostrum (second feeding).								
	2.783	Casein. 0.948	0.715	0.756	0.055		0.285	0.034

held from a calf for some hours after birth the globulin fractions have not appeared in the blood in appreciable amounts and no protein has appeared in the urine, but upon the subsequent ingestion of colostrum these proteins have appeared in the blood and urine. (*c*)

The proteins in the urine consist of the euglobulin, pseudoglobulin I and II, and albumin fractions.

There is but one point which needs particular discussion and that is with regard to the albumin which appears in the urine with the globulin fractions and in many cases in relatively large amounts. The direct absorption of *albumins* by the new-born calf is not so

TABLE V.

Calf 1034. Fed Blood Serum.

Calf was born Apr. 9, at 1.30 p.m. At 4.35 p.m., it was fed 1,000 cc. of blood serum of a cow and at 10.30 p.m., 300 cc. of blood serum.

Urine.—Protein was present in the urine collected at 5.20 a.m. on Apr. 10, and again at 4.45 p.m. The urine collected at 5.30 a.m. on Apr. 11, showed only a faint cloudiness, negative.

Sample collected.	Total N.	Serum N.	Euglobulin N.	Pseudoglobulin N.		Total globulin N.	Albumin N.	Nonprotein N.
				I	II			
Blood.								
Apr. 9, 4.25 p.m.		0.676	0.000	0.000	0.149	0.149	0.468	0.059
Fed blood serum at 4.35 p.m.								
Apr. 9, 10.30 p.m.		0.969	0.191	0.090	0.165	0.446	0.477	0.046
" 10, 9.35 a.m.		0.761	0.026	0.106	0.140	0.272	0.430	0.059
" 24		0.833	0.030	0.051	0.144	0.225	0.553	0.055
Urine.								
Apr. 10, 5.20 a.m.	1.131		0.037	0.066	0.011	0.114	0.036	0.913
Blood serum (cow).								
		1.398	0.151	0.216	0.150	0.697	0.504	0.056

strikingly demonstrable as the absorption of the globulin fractions because albumins are already present in the blood of a calf. An examination of all of our data relating to the absorption of protein by new-born calves does not disclose a definite increase in albumin coincident with the accumulation of globulins in the blood. There are cases in which the data might be interpreted as indicating a slight increase in albumin but there are other cases in which such a change is not evident.

A satisfactory explanation of the presence of the albumin fraction in urine is not at hand. In the formulation of any theory it is necessary to remember that the quantity of albumin in colostrum is roughly only about twice that found in milk. When milk is fed an appreciable amount of albumin does not appear in the urine. In one experiment in which the ratio of albumin to globulin in the ingested colostrum was 1:5, the ratio in the urine was 1:1. In this case the possible interference of urea was essentially eliminated. This fact is difficult to reconcile with the simple assumption, which appears to be evident in the case of the globulins, that there is a permeability of the kidney as well as the gastrointestinal tract for a short time after birth and that the globulin excreted in the urine arises unchanged from the globulin of the colostrum. A possible explanation might be that the albumin is derived, in part at least, from the absorbed globulin. Such an explanation involves theories with regard to the relation between the various protein fractions for which there is comparatively little satisfactory evidence. Evidence which we have presented for the calf (4) and other unpublished data indicate that variations in albumin in the blood are relatively independent of the variations in the globulin fractions.

It is apparent that the absorption of unchanged protein and the excretion of protein in the urine appear to be intimately related. On the other hand, at the time the absorption and excretion of protein has ceased relatively large amounts of the euglobulin and pseudoglobulin I fractions are still present in the blood. These fractions continue to decrease over a period of 4 to 6 weeks until the concentration of the blood of the post-colostrum phase of the calf is reached (4). This final decrease is independent of the excretion of protein in the urine of the new-born which lasts only a few days.

The cause of the albuminuria in the young has not, so far as we can find, been ascribed hitherto to the direct absorption and excretion of the proteins of colostrum. The bearing of this view on the interpretation of the literature has been briefly touched upon in a preceding paper of Smith and Little (1).

In a previous study of the fat content of the feces of young calves (5) we showed that the feces of the young calf which were passed a few days after birth contained considerable amounts of protein and

that the proteins, euglobulin, pseudoglobulin, and albumin, were present. A survey of the total nitrogen determinations of feces presented at that time on calves which had not received colostrum and on those which had received colostrum shows that the total fecal nitrogen of the former set of calves was lower than for the latter set. At about the same time we separated, by salting out with ammonium sulfate, from the urine of a calf 1 day old, euglobulin, pseudoglobulin, and albumin. The evidence presented in the preceding papers and in this paper indicates that the protein of the feces soon after birth probably has its origin in the high protein-containing colostrum ingested at birth.

SUMMARY.

The coincidence of the appearance of euglobulin and pseudoglobulin I in the blood and urine of the new-born calf following the ingestion of colostrum and the absence of these proteins when milk is fed support the inference that the proteinuria of the new-born calf is due to the ingestion of colostrum. In this passage the proteins are in part unaltered.

The absorption and excretion of the proteins of blood serum have been observed.

The high protein content of the feces of young calves—during the first few days—is to be ascribed, in part at least, to ingested colostrum.

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THE EFFECT OF INORGANIC SALTS ON THE CHEMICAL CHANGES IN THE BLOOD OF THE DOG AFTER OBSTRUCTION OF THE DUODENUM.

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A fundamental chemical change in the blood of the dog after high intestinal obstruction is a fall in chlorides.[1] The characteristic increase in non-protein nitrogen does not begin and the chemical evidence of toxemia does not appear until the chlorides are well depleted. These findings indicate that the rôle of the chlorides may be protective and they suggest the use of chlorides as a therapeutic measure.

It has been found that by supplying an abundance of sodium chloride from the onset of the obstruction of the intestine, toxemia may be entirely prevented.[2] One dog so treated lived 28 days without developing it. Untreated dogs die in 4 days on an average. Distilled water when given alone was found to hasten the fatal outcome. Solutions of glucose and sodium bicarbonate exerted no protective action.

The chemical changes reported as characteristic of intestinal obstruction in the dog have been found to occur also in intestinal obstruction in man.[3] Here also chlorides would appear to have great therapeutic value.

The experiments reported herewith have been made to determine if possible whether inorganic salts, other than sodium chloride, alter the course of the toxemia following obstruction of the duodenum.

[1] Haden, R. L., and Orr, T. G., *J. Exp. Med.*, 1923, xxxvii, 365.

[2] Haden, R. L., and Orr, T. G., *J. Exp. Med.*, 1923, xxxviii, 55.

[3] Haden, R. L., and Orr, T. G., *Surg., Gynec. and Obst.*, 1923, xxxvii, 465.

Method.

The animals have been kept in metabolism cages and given no food during the course of the experiments. All operations were done under ether anesthesia with aseptic technique. Water was allowed *ad libitum*. Blood for chemical analysis was obtained from the jugular vein before operation and at 24 hour intervals thereafter until the death of the animal.

The duodenum was obstructed by section about 8 inches from the pylorus and inversion of the cut ends. The obstruction was thus rendered complete in every instance. An autopsy was always performed. Peritonitis was never a complicating factor.

The non-protein nitrogen, sugar, and creatinine have been determined by the method of Folin and Wu.[4] The protein-free filtrates were made by a modification of the original Folin-Wu technique described by one of us.[5] The CO_2-combining power has been determined by the method of Van Slyke and Cullen,[6] urea nitrogen by the Van Slyke and Cullen modification of the Marshall method,[7] amino-acid nitrogen by the method of Folin,[8] uric acid by Benedict's method,[9] and the blood chlorides on the tungstic acid filtrate in the manner suggested by Gettler.[10]

The animals were each given 40 cc. of M/7 solution of some salt per kilo subcutaneously daily except as otherwise indicated.

EXPERIMENTAL.

Two animals were given sodium chloride in glucose solution to determine whether the glucose would increase the duration of life. The treatment instituted is indicated in Table I. One dog died on the 22nd day and the other on the 24th day after operation. Neither dog developed the chemical or clinical evidence of toxemia. These results confirm those previously reported concerning the protective action of sodium chloride in intestinal obstruction. The glucose solution evidently did not increase the protective value of the solution, since the results are similar to those obtained with sodium chloride solution alone.

The results following the administration of other chlorides are shown in Table II. Potassium, ammonium, calcium, and magnesium

[4] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
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[8] Folin, O., *J. Biol. Chem.*, 1922, li, 377.
[9] Benedict, S. R., *J. Biol. Chem.*, 1922, li, 187.
[10] Gettler, A. O., *J. Am. Med. Assn.*, 1921, lxxvii, 1652.

TABLE I.

Obstruction of the Duodenum. Blood Findings during Treatment with Sodium Chloride and Glucose.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.	CO_2-combining power.	Treatment (40 cc. per kilo).
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
1	Day before.	34.5	17.8	3.2	1.7	12.2	91	420	38.1	M/6.88 NaCl in 5 per cent glucose.
	0	39.5	10.3	2.7	2.4	16.0	74	430	34.3	" " " " " " "
	1	31.3	10.7	2.2	2.3	14.0	37	400	33.7	" " " " " " "
	2	24.6	8.9	2.2	1.3	9.7	80	380	47.5	" " " " " " "
	3	31.9	10.3	1.9	1.1	7.6	74	350	47.5	" " " " " " "
	4	38.9	21.9	1.7	1.1	6.6	74	270	62.6	M/2.93 " " " " " "
	5	27.3	11.2	2.2	1.5	6.0	89	360	55.1	" " " " " " "
	6	26.8	9.3	1.4	1.2	5.0	69	400	63.6	" " " " " " "
	7	38.0	12.6	1.3	1.3	10.4	52	310	52.2	M/6.88 " " " " " "
	8									" " " " " " "
	9	28.5	14.9	1.3	1.1	3.7	80	410	57.9	" " " " " " "
	10									" " " " " " "
	11	48.2	21.5	1.6	1.2	3.8	47	510	53.1	" " " " " " "
	12	34.9	11.7	1.7	1.2	4.4	87	500	50.4	" " " " " " "
	13	27.7	9.9		1.6	6.2	81	470	64.4	" " " " " " "
	14									" " " " " " "
	15	30.0	15.4	1.8	1.6	6.2	110	400	77.2	" " " " " " "
	16	26.8	7.0	1.4	1.0		69	350	77.7	M/2.93 " " " " " "
	17									" " " " " " "
	18	27.0	7.0	1.4	1.1		43	330	67.3	" " " " " " "
	19									" " " " " " "
	20	22.4	10.5	1.5	1.3	4.6	59	390	60.6	" " " " " " "
	21	33.3	14.5	0.8	1.2	4.3	47	400	75.0	M/5.85 " " " " " "
	22	53.5	29.4	2.0	1.1	5.8	58		48.6	" " " " " " "
	23									" " " " " " "
	24									Died.
2	Day before.	40.0	14.0	2.2	1.8	14.6	69	510	23.0	
	0	34.9	12.6	1.8	2.0	13.4	52	430	40.0	M/6.88 NaCl in 5 per cent glucose.
	1	30.3	7.0	1.5	2.1	8.7	54	455	31.8	" " " " " " "
	2	33.3	6.1	2.6	1.0	11.2	111	380	45.7	" " " " " " "
	3	33.3	7.0	1.4	1.1	5.7	46	400	41.9	" " " " " " "
	4	37.5	10.7	2.6	1.1	6.2		360	43.8	M/2.93 " " " " " "
	5	37.5	13.5	0.8	1.1	7.2	35	390	41.9	" " " " " " "

TABLE I—*Concluded.*

Dog No.	Day after operation.	Blood. Amount per 100 cc. Non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.	CO_2-combining power.	Treatment (40 cc. per kilo).
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
2	6	36.6	6.5	0.7	1.1	10.4		480	40.9	M/6.88 NaCl in 5 per cent glucose.
	7	35.3	8.9	0.6	1.1	7.7		470	29.9	" " " " " " "
	8									" " " " " " "
	9	38.9	18.6	0.7	1.1	3.8	37	470	52.2	" " " " " " "
	11	42.8	22.4	0.7	1.1	8.5			46.7	
	12	53.5	24.8	0.7	1.3	5.3	52	540	49.4	" " " " " " "
	13	30.0	16.8	0.7	1.2	6.2	41	500	68.1	" " " " " " "
	14									" " " " " " "
	15	31.6	16.8	0.7	1.9	6.6	63	510	65.2	" " " " " " "
	16	28.0	13.3	0.7	1.0		39	450	59.8	" " " " " " "
	17	37.5	15.4	0.8	1.1		55	430	69.2	" " " " " " "
	18									" " " " " " "
	19									" " " " " " "
	20	27.3	12.6	1.0	1.2	5.4	45	470	68.0	M/5.85 " " " " " "
	21	35.3	17.8	1.0	1.3	6.1	43	440		" " " " " " "
	22									Died.

chlorides were found to have no protective value. The animals all showed a rapid fall in blood chlorides and died quickly. Ammonium chloride produced a marked acidosis as shown by the rapid fall in CO_2-combining power. The fact that ammonium chloride produces an acidosis has been recently pointed out by Haldane.[11] He explains this result as due to the rapid conversion of ammonia to urea, leaving the HCl free to neutralize the body alkali.

One dog was given M/7 potassium iodide and a second M/7 sodium iodide. Both showed a marked fall in chlorides and a rise in non-protein nitrogen, dying within 48 hours (Table III).

The findings on treatment with sodium bromide are interesting. M/14 solution had no protective value. Two dogs were given M/7

[11] Haldane, J. B. S., *J. Physiol.*, 1921, lv, 265.

TABLE II.

Obstruction of the Duodenum. Blood Findings during Treatment with Potassium, Ammonium, Calcium, and Magnesium Chloride.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.	CO_2-combining power.	Treatment (40 cc. per kilo).
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
3	0	32.6	15.4		1.3	7.1	86	470	37.2	M/7 KCl
	1	29.7	11.7		1.1	7.1	130	440	41.9	" "
	2	60.0	32.2	1.1	2.7	18.6	114	340	40.0	" "
	3									Died.
4	0	24.0	11.2	1.2	1.2	4.3	79	530	24.5	M/7 KCl
	1	62.5	33.6		1.8	5.5	72	475	29.1	" "
	2									Died.
5	0	27.7	9.1		1.3	5.4	91	470	34.3	M/7 NH_4Cl
	1	53.5	26.1		1.3	5.9	121	390	15.0	" "
	2									Died.
6	0	30.3	14.0		1.1	5.7	93	490	37.2	M/7 NH_4Cl
	1	27.3	11.2		1.1	5.7	104	440	26.8	" "
	2	110.8	64.4		3.8	14.0	125	400	10.7	" "
										Died.
7	0	43.5	21.0	1.2	2.0	11.2	116	470	37.2	M/14 $CaCl_2$
	1	31.6	14.7	1.2	2.1	16.4	105	450	30.5	" "
	2									Died
8	0		29.89	2.9	1.0	5.9	107	480		M/35 $CaCl_2$
	1	94.0	31.6	2.9	1.1	7.6	115	360	41.9	" "
	2									Died.
9	0	30.0	11.7		1.6	5.1	77	480	31.0	M/7 $MgCl_2$
	1	47.6	19.6	1.6	2.3	7.4	93	435	31.0	" "
	2	111.0	68.7	2.2	3.8	6.8	125	370	28.7	" "
	3									Died.
10	0	35.3	14.5	1.7	1.3	7.6	80	450	33.2	M/7 $MgCl_2$
	1	31.6	15.4	3.8	1.3	13.2	105	390	33.8	" "
	2	147.0	72.8	2.9	1.3		95	420	25.8	" "
	3									Died.

TABLE III.

Obstruction of the Duodenum. Blood Findings during Treatment with Iodide and Bromide.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.	CO_2-combining power.	Treatment (40 cc. per kilo).
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
11	0	31.0	14.0					450	27.7	M/7 KI
	1	22.4	8.4		1.4	5.0	85	350	41.9	" "
	2	57.5	24.5		1.7	8.1	93	420	45.7	" "
	3									Died.
12	0	55.5	21.5	2.7	1.6	6.6	69	465	20.8	M/7 NaI
	1	67.5	39.2	2.8	1.2	11.6	83	365	38.4	" "
	2									Died.
13	0	38.5	17.3	1.1	1.3	6.2	79	480	38.1	M/7 NaBr
	1	37.3		1.7	1.2	7.1	125	460	36.2	" "
	2	37.0	14.0	1.4	1.1	8.6	100	440	45.7	" "
	3	36.6	13.1	1.4	1.1	7.1	149	400	58.9	" "
	4	34.9	12.1	1.4	1.2	7.0	149	400	61.7	" "
	5	31.6	13.7	1.3	1.2	6.5	149	470	57.0	" "
	6									Died.
14	0	35.3	15.9	3.0	1.0	7.7	139	430		M/14 NaBr
	1	51.6	18.1	2.3	1.1	10.7	111	400	28.7	" "
	2	97.3		2.7	1.1	8.0	130	330	55.7	" "
	3	81.5	43.5	2.8	1.1	6.2	91	260	64.5	" "
	4	177.0	81.5	2.9	1.1	5.3	139	220	80.6	" "
	5	200.0	80.0	3.3	1.1	7.0	145	310	63.7	" "
	6									Died.
15	0	25.0	16.4	1.8	1.6		47	470	32.2	M/7 NaBr
	1	30.0	19.6	1.8	1.3	4.9	33	430	26.3	" "
	2	36.6	13.1	1.7	1.5	5.3		440	43.8	" "
	3	30.0	13.5	2.6	1.2	5.0		455	47.6	" "
	4	23.6	13.5	2.0	1.2	5.1	35	440	39.3	" "
	5	24.8	10.5	2.0	1.1	5.8	46	400	34.3	" "
	6	141.0	53.9	2.6	1.2	6.1	50	420	43.0	" "
	7	101.0	47.5	2.1	1.0	5.3	71	410	47.7	" "
	8	103.0	53.2	2.5	1.0	5.0	40	420	41.2	" "
	9									Died.

TABLE IV.

Obstruction of the Duodenum. Blood Findings during Treatment with Sulfate, Phosphate, and Citrate.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.	CO_2-combining power.	Treatment (40 cc. per kilo).
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
16	0	34.5	7.0	4.0	1.2	6.6	53	470	35.3	M/7 Na_2SO_4
	1	63.2	20.6	4.4	1.3	7.0	74	300	61.7	" "
	2									Died.
17	0	38.9	19.6	2.7	1.2	8.7	69	490	21.1	M/14 $MgSO_4$
	1	29.4	11.2	2.1	1.5	7.3	103	400	49.4	" "
	2	55.0	27.1	3.2	1.4	7.3	98	320	47.5	" "
	3 (a.m.)	90.0	49.1	3.0	2.0	10.4	98	200	40.0	" "
	3 (p.m.)	121.0	78.2	1.7	2.3	10.0	149	200	37.2	" "
	4									Died.
18	0	22.8	13.1	2.3	1.3	8.5	79	420	38.4	M/7 $MgSO_4$
	1	46.0	15.5	3.0	1.7	9.3	100	410	47.7	" "
	2 (a.m.)	102.5	57.4	3.8	3.5	9.3	104	370	45.7	" "
	2 (p.m.)	163.0	82.7	4.4	4.0	10.4		460	19.8	" "
	3									Died.
19	0	32.6	10.3	2.2	1.7	8.2	93	520	40.1	M/14 $MgSO_4$
	1	40.0	14.5	3.1	1.6	9.3	86	360	42.0	" "
	2 (a.m.)	192.0	78.2	3.8	3.5	11.2	111	300	26.8	" "
	2 (p.m.)	200.0	113.5	3.8	3.5	14.0		310	21.7	" "
	3									Died.
20	0	28.5	9.8		1.5	6.3	87	460	42.8	M/7 sodium citrate.
	1									Died during night.
21	0	46.0	10.3	2.3	1.2	6.1	67	510	39.0	M/7 NaH_2PO_4
	1	71.3	34.7		1.3		54	440	59.8	" "
	2	85.3	39.1	2.1	1.5	7.1	54	110	42.0	" "
	3									Died.
22	0	55.0		3.1	1.2	10.0	192	460	10.7	M/7 Na_2HPO_4
	1									Died during night.

TABLE V.

Obstruction of the Duodenum. Blood Findings during Treatment with Pilocarpine and Atropine.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.	CO_2-combining power.	Treatment.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
23	0	23.0	12.6	2.0	1.1	6.5	77	450	41.9	3 cc. of 0.1 per cent pilocarpine per kilo daily.
	1	59.0	29.9	2.3	1.5	7.6	103	370	40.9	
	2	121.0	78.2	2.0	1.4	13.4	109	310	41.9	
	3									Died.
24	0	42.6	12.1	2.2	1.3	6.3	93	460	45.7	3 cc. of 0.1 per cent pilocarpine per kilo.
	1									Died during night.
25	0	40.0	17.3	1.4	1.4	6.2	76	430	41.0	1 cc. of 0.1 per cent atropine per kilo daily.
	1	70.0	32.2	2.0	1.3	7.6	44	340	35.3	
	2									Died.
26	0	35.3	7.5	1.8	1.1	6.5	76	470	40.0	1 cc. of 0.1 per cent atropine per kilo daily.
	1	42.8	12.6	1.8	1.2	7.2	111	400	55.1	
	2	106.0	47.6	2.5	1.2	8.3	133	375	62.6	
	3									Died.

solution. One died on the 6th day without an increase in non-protein nitrogen. The blood chlorides had remained at almost the normal level. A second dog showed no increase in non-protein nitrogen until the 6th day and died on the 9th day. The blood chlorides showed relatively little change.

M/7 solution of sodium sulfate, magnesium sulfate, sodium citrate, monosodium phosphate, and disodium phosphate respectively had no protective action (Table IV). The dog given the M/7 monosodium phosphate showed a blood chloride determination of 110 mg. per 100 cc. This is the lowest figure met with in this series.

To determine whether autonomic drugs would influence the course of events following obstruction of the duodenum, two dog were treated

with pilocarpine and two with atropine (Table V). One animal lived less than 24 hours, one less than 48 hours, and two died within 72 hours. All dogs from which specimens of blood were obtained after operation showed the chemical changes characteristic of the toxemia of intestinal obstruction.

DISCUSSION.

The experiments here reported offer further proof of the changes previously described as characteristic of intestinal obstruction. The results obtained in the two dogs treated with sodium chloride emphasize again the great protective value of this salt against the toxemia. The addition of glucose did not enhance the value of the salt solution. The fact should be mentioned that one dog treated with the glucose-sodium chloride solution was jaundiced for a number of days before death and at autopsy showed marked atrophy of the liver.

. Iodides seemingly hasten the action of the toxic body, while sodium bromide appeared to exert an inhibitory influence. One animal given sodium bromide lived 5 days and showed no increase in non-protein nitrogen before death. Another died on the 9th day with only a moderate increase. The other salts used had no protective value. Atropine and pilocarpine likewise did not alter the course of the intoxication.

SUMMARY.

The chemical changes in the blood of dogs treated with various inorganic salts after obstruction of the duodenum are reported.

Two dogs treated with sodium chloride survived approximately six times as long as the average untreated animal, one living 22 days, the other 24 days.

Ammonium chloride was found to produce an acidosis. The administration of potassium chloride, calcium chloride, and magnesium chloride did not prevent the usual rise in non-protein nitrogen and fall in chlorides, and the fatal outcome.

Iodides seemingly hasten the toxic process. Sodium bromide appears to have an inhibitory action upon it, but much less than that of sodium chloride.

Sodium sulfate, magnesium sulfate, sodium citrate, monosodium phosphate, and disodium phosphate failed to alter the course of the intoxication.

Atropine and pilocarpine were without therapeutic value in preventing the changes characteristic of intestinal obstruction.